Aspects of genetic and environmental control of phase II metabolism

Fordyce, Karen

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ASPECTS OF GENETIC AND ENVIRONMENTAL CONTROL OF PHASE II METABOLISM

Submitted by KAREN FORDYCE B.Sc.

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ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

Dedication

To my parents and Adrian, with love and many thanks for their continual support and encouragement.

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ABBREVIATIONS

ASA	acetyl salicylic acid
dpm	disintegrations per minute
E_2	estradiol
EE_2	ethinyl estradiol
E_2G	estradiol glucuronide
E_2S	estradiol sulphate
FSH	follicle stimulating hormone
HPLC	high performance liquid chromatography
LH	luteinising hormone
OCS	oral contraceptive steroids
P	paracetamol
PG	paracetamol glucuronide
PS	paracetamol sulphate
SA	salicylic acid
SUA	salicyluric acid

SUMMARY

1. The glycine conjugate of salicylic acid (SA) and the glucuronide and sulphate conjugates of paracetamol were examined in a population of healthy volunteers (n = 150 and 99 respectively) following therapeutic doses of aspirin (600 mg) and paracetamol (1 g). These 2 population studies enabled the genetic and environmental effects of three phase II metabolic pathways (glycine, glucuronide and sulphate conjugations) to be investigated.

A significant relationship between urine pH and SA excretion was noted. Age and body weight were found to have no effect upon the 3 conjugation pathways studied. Sex differences were found in the glycine conjugation of salicylate but not in the glucuronide and sulphate conjugations of paracetamol. The effects of oral contraceptive steroid (OCS) use and smoking on the 3 conjugation pathways were also studied. Glycine conjugation of salicylate was not affected by either. Paracetamol glucuronidation was increased by OCS and smoking whereas paracetamol sulphation was found to be decreased only by OCS use.

The population studies for both aspirin and paracetamol showed that glycine, glucuronide and sulphate conjugation are not subject to genetic control.

2. The metabolism of estradiol was studied in OCS users and non-users in order to examine the effects of the single environmental

factor, OCS, on endogenous metabolism. The plasma concentration of endogenous estradiol (E_2) and the urinary excretion of its metabolites, free E_2, E_2-glucuronide and E_2-sulphate were all found to be decreased by OCS use. In addition, the metabolism of estradiol may be altered by OCS use; the results indicating that the oxidation of estradiol may be decreased and the glucuronidation may be increased.

3. A parallel study to 2. was performed in rats where female rats were chronically pretreated with ethinyl estradiol (EE_2) and the effects on paracetamol and estradiol metabolism were examined. Paracetamol glucuronidation was found to be induced by EE_2 pretreatment whilst paracetamol sulphation was subject to a delayed decrease. Hence, it is proposed that EE_2 may be the component of the combined OCS responsible for the increase in paracetamol glucuronidation found in females. Similarly, the urinary excretion of total estradiol was found to be increased by EE_2 pretreatment although the small intestine content of estradiol was found to be decreased, indicating that EE_2 may reduce the enterohepatic circulation of estradiol.

CHAPTER ONE

INTRODUCTION

1.1 DRUG METABOLISM

Many drugs upon entering the body are transformed into other substances. The result of these processes is that the polarity of the compounds are increased so that they are readily excreted from the body in the urine and bile. Williams (1971) summarised the possible fate of drugs in the body as follows:

1. Drugs can undergo enzyme-catalysed transformations.
2. Drugs can be excreted unchanged (e.g. cyclamate; decamethonium).
3. Drugs can undergo a spontaneous reaction when given the appropriate physical conditions, such as pH (e.g. thalidomide)

Most drugs fall into the first category and the enzymes responsible for the transformations are found mainly in the liver, and to a lesser extent in other tissues including the intestine, kidney and lung. The process of biotransformation or metabolism is regarded as occurring in 2 phases, and can be summarised as follows:

Phase I Metabolism

Phase I involves oxidation, reduction and/or hydrolysis and can result in

a) inactivation of the drug (e.g. oxidation of tolbutamide)
b) conversion of an inactive drug into an active metabolite (e.g. prontosil → sulphonilamide)
c) conversion of one active drug into another (e.g. phenacetin → paracetamol).

Phase I reactions usually introduce chemical groups such as -OH, -COOH or $-NH_2$ which allow phase II metabolism or conjugation to occur. However, if a drug contains suitable groups, it can undergo phase II metabolism directly, as no phase I reaction is necessary. A drug may undergo only phase I metabolism as in the case of ethanol, which is oxidised mainly to CO_2. In general, phase I metabolism results in the polarity of the compound being increased, but the activity still remains.

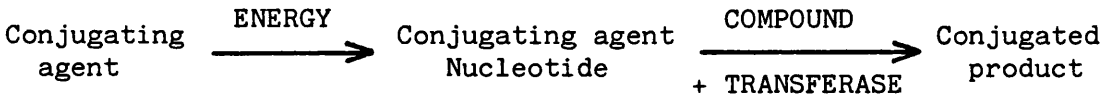
Within phase I metabolism there are 3 major reactions, namely oxidation, reduction and hydrolysis and of these oxidation is the most common. All oxidation reactions require $NADPH_2$ and O_2 and the enzymes responsible are usually found in the endoplasmic reticulum or microsomal fraction of the hepatic cell. Similarly, reduction involves hepatic microsomal enzymes whilst the enzymes responsible for hydrolysis are widely distributed throughout the body.

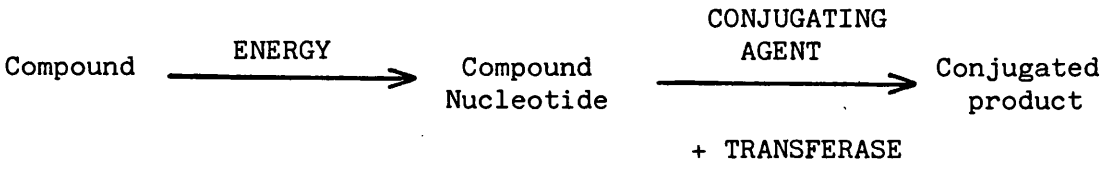
Phase II Metabolism

Phase II metabolism consists of synthetic reactions where the parent drug or its phase I metabolite is coupled with an endogenous substrate. Unlike phase I it generally results in termination of biological activity.

Within phase II metabolism there are 10 major reactions, 8 of which occur in man. These reactions involve the coupling of a compound containing a suitable chemical group (such as OH, NH_2 and COOH) with an endogenous molecule such as glycine or glucuronic acid. This endogenous molecule or conjugating agent is usually derived from the body's carbohydrate or protein sources.

As conjugations are synthetic reactions the majority require a source of energy which is usually supplied by adenosine triphosphate (ATP). ATP is required to form an intermediate activated nucleotide which is then catalysed by the transferring enzyme into the conjugation product. The intermediate nucleotide can be derived from either the conjugating agent or the compound undergoing conjugation as shown below:





Of the conjugation reactions that occur in man, glucuronic acid conjugation is the most widespread and it is the only phase II reaction to take place in the microsomal fraction of the liver. Of

the other major phase II reactions, glycine conjugation occurs in hepatic mitochondria whilst sulphation occurs in the kidney and the intestinal mucosa as well as the soluble fraction of the hepatic cell. Similarly, acetylation has a wide distribution throughout the body (including the liver, lung and spleen) whilst the majority of mercapturic acid synthesis occurs in the kidney.

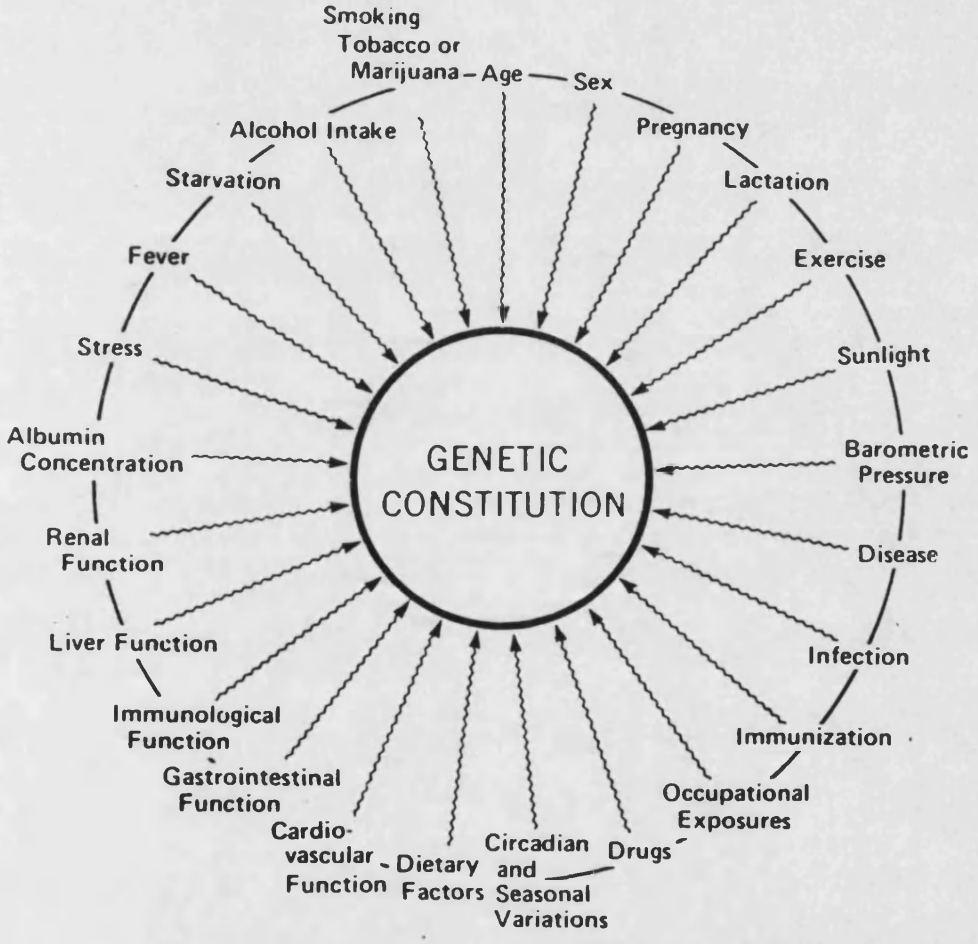
Although foreign compounds such as drugs and some food substances undergo metabolism upon entering the body, the same metabolic pathways are used by a variety of endogenous compounds. These include bilirubin, bile salts and the steroid hormones; by metabolism they lose their biological activity and/or are excreted from the body.

1.2 FACTORS AFFECTING DRUG METABOLISM

Large variations in drug response and therapeutic outcome may be observed if the same dose of a drug is administered to different individuals. One major factor contributing to interindividual differences is a marked variation in the capacity to metabolise drugs, which in turn is due to variations in the nature and amounts of enzymes responsible for drug metabolism. These enzymes and their activities are controlled by a complex interplay of genetic and environmental factors. Fig. 1.1 (Vesell, 1982) shows the known or suspected host factors that may influence drug response in man. The multiplicity of interaction amongst host factors and the modulation of these host factors by genetic constitution is also depicted.

FIGURE 1.1

The multiplicity of factors that may influence drug response in man.



This circular design suggest the multiplicity of either well-established or suspected host factors that may influence drug response in man. A line joins all such factors in the outer circle to indicate their close interrelationship. Arrows from each factor in the outer circle are wavy to indicate that effects of each host factor on drug response may occur at multiple sites and through different processes that include drug absorption, distribution, metabolism, excretion, receptor action, and combinations thereof.

Vesell 1982
Clin. Pharmacol. Ther. 31(1): 1.

a) Genetic Control of Drug Metabolism

There are 2 types of genetic control of metabolic reactions in man. In one type, a frequency distribution histogram relating incidence to metabolic response shows a continuous (normal) distribution with wide differences between the 2 extremes. This is due to the metabolic reaction being controlled by a number of genetic and environmental factors, and so the control is polygenic and multifactorial. In the second type a discontinuity of metabolic response is obtained whereby the frequency distribution histogram gives a bimodal or trimodal distribution. This indicates monogenic control with different individual modes corresponding to different allele pairing (Ritchie *et al.*, 1980). It is this second group which complies with the definition of genetic polymorphism:

"Genetic polymorphism is a type of variation in which individuals with sharply distinct qualities co-exist as normal members of a population". (Ford, 1965).

The condition is also defined as "the occurrence together in the same habitat of 2 or more discontinuous forms or phases of a species in such proportions that the rarest of them cannot be maintained by recurrent mutation". (Ford, 1942).

Many drugs undergo extensive metabolism which terminates biological activity when given orally, which affects the bioavailability of the active drug and the response obtained. The major factor responsible for such metabolism is hepatic oxidation which has been

found to be under genetic control. A good example of polymorphic drug oxidation is observed with the antihypertensive drug debrisoquine.

Polymorphism of Oxidation

The optimal dose requirements of debrisoquine have shown marked interindividual differences and these have been found to be due to differences in ability to metabolise the drug (Angelo *et al.*, 1975). Further studies, showing a daily dosage range of 10 to 360 mg in 120 hypertensive subjects, have found a positive correlation between the hypotensive response to debrisoquine and the amount of unchanged drug excreted in the urine (Silas *et al.*, 1977).

The major metabolic pathway of debrisoquine, 4-hydroxylation, is under genetic control (Mahgoub *et al.*, 1977). The metabolic ratio between the urinary excretion of the parent drug and its principal metabolite, 4-hydroxydebrisoquine, gave a bimodal distribution with 2 distinct phenotypes of extensive and poor metabolisers. Approximately 7 - 9% of the U.K. population are poor metabolisers of debrisoquine although this frequency appears to be different in other ethnic groups (Kalow *et al.*, 1982). Family studies have shown that this phenotype is controlled by an autosomal recessive gene (Mahgoub *et al.*, 1977). The genotype responsible for deficient 4-hydroxylation also controls a wide variety of phenotypes characterised by deficient metabolism of numerous drugs. These include sparteine, nortriptyline, phenytoin, guanoxan and phenacetin and certain β-blocking drugs such as metoprolol,

bufuralol and timolol. At least 22 drugs have been found to undergo the same hepatic drug oxidising pathway through which debrisoquine passes.

Polymorphism of Hydrolysis

The cytochrome P_{450} responsible for the oxidation of debrisoquine and other related compounds is not the only enzyme system in man to be under genetic control. Hydrolysis is also subject to polymorphism as seen with the muscle relaxant, succinylcholine. As the enzyme responsible for succinylcholine hydrolysis, pseudocholinesterase, is found in the plasma and so is readily accessible, an *in vitro* test is performed to determine the dibucaine number which represents the affinity of the enzyme for succinylcholine. A trimodal distribution of the dibucaine number is obtained and the three phenotypes are due to the autosomal and autonomous control of the hydrolytic enzyme. It is subjects who are homozygotes of the atypical pseudocholinesterase who are slow metabolisers of succinylcholine.

Polymorphism of Acetylation

Drugs which undergo N-acetylation, a phase II metabolic reaction, have also been found to show polymorphism. Both plasma isoniazid concentrations and urinary excretion of unchanged isoniazid gave a bimodal distribution with the 2 phenotypes of rapid and slow acetylation (Evans *et al.*, 1960). Family studies have shown that the slow N-acetylator phenotype is an autosomal homozygous recessive. As with cytochrome P_{450} polymorphism a variety of drugs

which under N-acetylation also exhibit deficient metabolism in a percentage of the population. These include dapsone, hydralazine, procainamide, phenelzine, nitrazepam and certain sulphonamides such as sulfapyridine and sulfadimidine. Approximately 38% of British Caucasians are slow acetylators but this percentage varies widely between ethnic groups.

The Significance of Polymorphism of Oxidation, Hydrolysis and Acetylation

Metabolism, phase I and/or phase II is responsible for the inactivation of many drugs. Therefore variations in metabolism can be a major influence on interindividual differences in responsiveness to drugs. This can be seen in the polymorphisms of oxidation, acetylation and hydrolysis where a drug's response is related to the phenotype of the subject. For example, poor oxidisers, when administered debrisoquine, are more likely to develop postural hypotension than extensive oxidisers. Similarly, upon administration of isoniazid,slow acetylators are more susceptible to its toxic effects such as peripheral neuritis, whilst slow hydrolysers are more prone to prolonged apnoea than rapid hydrolysers when given succinylcholine. Toxicity of pharmacogenetic origin occurs when subjects are given drugs which they are genetically unable to inactivate in the usual way, resulting in drug accumulation. Thus pharmacogenetics deals with inborn errors of metabolism involving defective enzymes that convert pharmacologically active drugs into inactive metabolites.

b) Environmental Control

The activity of drug metabolising enzymes can be altered by numerous environmental factors, although the majority of this research has concentrated on hepatic microsomal metabolism. A summary of the factors known to affect metabolism as stated by Williams (1972) is given below:

Species	Sex
Strain	Stress
Age	Temperature
Chronic administration	Time of day
Other drugs or foreign compounds	Season
Route of administration	Biliary excretion and entero-hepatic circulation
Disease	Gut flora
Diet	Altitude

Due to the large number of factors which have been proposed to affect drug metabolism, only those which are of particular relevance to the present work will be discussed further.

Other Drugs or Foreign Compounds

Chronic administration of one drug can result in decreased pharmacological activity of another drug by stimulating its inactivating metabolic pathway thus reducing its concentration at its active site(s). Such stimulation of metabolic enzymes is caused by the amount of enzyme being increased, which is the result of

either an increase in enzyme synthesis or a decrease in enzyme degradation. Enzyme stimulation is known as enzyme induction and when compounds capable of enzyme induction are given concomitantly with another drug, the metabolism and therefore the activity of the second drug are altered. Examples of drugs which are capable of causing enzyme induction include polycyclic hydrocarbons such as 3-methylcholanthrene and 3,4-benzpyrene which show considerable substrate specificity and barbiturates which are relatively non-specific. Conney and Burns (1962) have reviewed the enzyme induction of microsomal enzymes.

Age

The newborn are known to have a reduced capacity for drug metabolism, with the most prominent effect being in reduced glucuronyl transferase activity. This results in a reduced ability to conjugate bilirubin and transient hyperbilirubinaemia may occur. However, as the baby grows, the capacity for glucuronide conjugation increases.

There is now increasing evidence to show that drug metabolism is impaired in old age. The elderly have been found to have significantly reduced plasma clearance of several drugs including antipyrine, chlormethiazole, phenylbutazone and quinine, all of which undergo oxidation. For a review on metabolism and the elderly, see Crooks and Stevenson, 1977. Conjugation pathways including glycine, glucuronide and sulphate conjugation also appear to be affected by age. The plasma clearance of drugs which undergo phase II metabolism, such as aspirin and paracetamol, have also

been found to be significantly reduced in the elderly (Ho *et al.*, 1985; Triggs *et al.*, 1975 respectively).

Sex

Sex differences in drug metabolism have mainly been studied in rats and some phase I metabolic pathways have been found to be sex-dependent whilst others are relatively independent of sex (Kato, 1974). Similarly, recent studies have determined the effect of sex on oxidative drug metabolism in man with drugs such as antipyrine (Teunissen *et al.*, 1982), diazepam (Greenblatt *et al.*, 1980) and chlordiazepoxide (Roberts *et al.*, 1979) showing sex differences.

Disease and Diet

Changes in nutritional states and numerous pathological states such as liver and kidney disease, hormonal disturbances, tumour-bearing states and adjuvant arthritis are all capable of affecting hepatic metabolism. Phase I microsomal metabolic pathways are generally more susceptible to such changes in nutritional and pathological states but other pathways such as conjugation can be affected to a lesser extent. Kato (1977) provides an excellent review of the effects of disease and diet on drug metabolism.

In the present work, the metabolism of aspirin and paracetamol was studied in human volunteers in order to identify the environmental factors that may affect the major metabolic pathways of these 2

commonly-used compounds. These 2 studies were also performed to show whether the major metabolites of paracetamol and aspirin are subject to genetic control. A further study was performed in order to assess the effect of a single environmental factor; chronic use of oral contraceptive steroids, on endogenous estradiol metabolism in female volunteers.

1.3 ASPIRIN

The salicylates have been used for over 2000 years in the treatment of a variety of conditions. Salicylin was first isolated from willow bark in 1826 but it was not until 1893 that Felix Hoffman developed a commercial method for synthesising aspirin, thus bringing it into widespread use. Today the salicylates are available in hundreds of different forms and annual world consumption can be measured in billions of doses.

a) Mode of Action and Therapeutic Uses

Analgesic Effect

Salicylates are mild analgesics which are effective in relieving mild to moderate pain. Lim (1964) demonstrated that aspirin acts peripherally as opposed to morphine which has a central action. Salicylates are believed to cause pain relief by inhibiting the local synthesis and release of prostaglandins (Ferreira and Vane, 1974). They are not effective as analgesics in non-inflamed tissues where prostaglandins are not responsible for the pain. Thus, salicylates are effective in the dull throbbing pain of inflammation where prostaglandins apparently sensitise the nerve endings but are ineffective in sharp, stabbing pain caused by direct stimulation of the nerves.

Salicylates are an effective analgesic for pain of moderate intensity such as headache, myalgia, arthralgia, and dysmenorrhea. They are also particularly effective at controlling chronic post-operative pain or pain arising from inflammation.

Anti-inflammatory Effect

Salicylates modify or diminish the inflammation process, but are not capable of arresting it. Numerous mediators are known to be involved in inflammation including histamine, 5-hydroxytryptamine, slow releasing substance of anaphylaxis, bradykinin and prostaglandins. Salicylates exert their antinflammatory effect by inhibiting the synthesis and release of bradykinin and prostaglandins (Barnett *et al.*, 1982). The Anti-inflammatory effects of salicylates are used in the treatment of musculoskeletal disorders such as rheumatoid arthritis, osteoarthritis and alkylosing spondylitis. However, they only provide symptomatic relief; the progression of the disease is not arrested.

Antipyretic Effect

During inflammation, endogenous pyrogen is released which causes prostaglandin E_2 to be released from discrete sites within the brain, resulting in an elevation of body temperature. Salicylates are effective antipyretics as they inhibit prostaglandin production so preventing further increases in body temperature.

Antithrombotic Effect

Aspirin reduces platelet aggregation. Thromboxane A_2 induces platelet aggregation and prostaglandin I_2 inhibits platelet aggregation. If a suitably low dose of aspirin is given, selective inhibition of thromboxane A_2 occurs, resulting in decreased platelet aggregation.

Aspirin has been suggested as an antithrombotic agent to be used in

thrombotic diseases such as coronary artery disease, myocardial infarction and post-operative deep vein thrombosis. However, much controversy still exists regarding the efficacy of aspirin in this area.

b) Toxicity

The most common adverse effects of salicylates are associated with the gastrointestinal tract (Silvoso *et al.*, 1979) namely irritation of the gastric mucosa, which at high doses can result in exacerbation of peptic ulcer, dyspepsia, gastric bleeding and erosive gastritis. Salicylate induced gastric bleeding is painless and although it frequently leads to blood loss in the stools, iron-deficiency anaemia occurs only occasionally.

Central nervous system toxicity is also found with salicylates. Tinnitus, deafness, headache, and dizziness often occur at high doses but disappear on reduction of the dose.

High doses of aspirin have been associated with nephrotoxicity. Transient shedding of renal tubular cells, alteration in urate clearance and reversible renal functional impairment have been found. However, severe renal failure is most rare, although nephrotoxicity is associated with prolonged use. Similarly, high doses of salicylates may be capable of causing liver damage. The hepatic damage takes up to several weeks to occur and results in elevated levels of plasma transferases.

Aspirin allergy is found in approximately 1% of the population and symptoms include urticaria-angiodema, bronchospasm, severe rhinitis

or shock. This hypersensitivity is more common in asthmatics or other individuals with allergies but the aspirin allergy is not thought to be an immunological reaction.

1.4 METABOLISM OF ASPIRIN

Absorption and Distribution

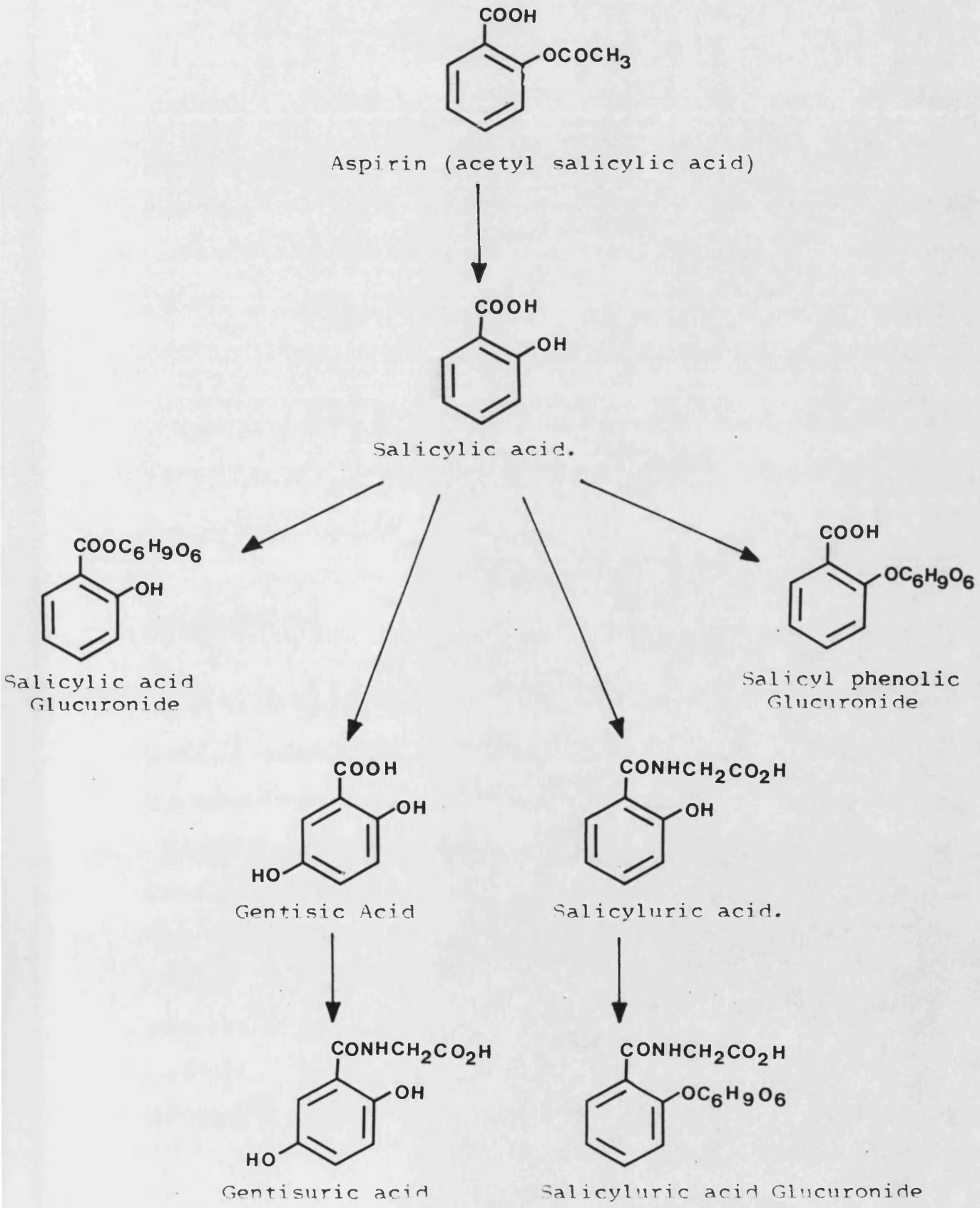
Upon oral administration, aspirin is rapidly absorbed from the stomach and more extensively from the jejenum (due to the large surface area). The absorption occurs by a passive diffusion of non-ionised, lipophilic molecules (aspirin and salicylic acid) across the gastro-intestinal membrane.

Approximately 60% of the aspirin dose reaches the systemic circulation intact (Rowland *et al.*, 1972). The remaining dose undergoes first-pass hydrolysis by esterases found in the intestinal wall, liver and plasma. A peak plasma concentration of aspirin is obtained within approximately 25 minutes for soluble aspirin preparations (Rowland *et al.*, 1972) but it declines rapidly as it is hydrolysed to salicylic acid.

Aspirin (ASA) and salicylic acid (SA) are both partially bound to serum proteins with SA being more extensively bound than ASA. However, the protein binding of both ASA and SA differs from other drugs in that it is dose-dependent; the fraction of the dose bound decreases as the serum concentration increases (Paulus *et al.*, 1971). They are both distributed throughout most body fluids and have been detected in spinal, synovial and peritoneal fluids, saliva and breast milk.

FIGURE 1.2

Metabolic Pathways of Aspirin.



Metabolism and Excretion

Aspirin is rapidly converted to salicylic acid by esterases found in the plasma, liver and intestinal wall. The majority of salicylic acid undergoes further metabolism but some is excreted unchanged. Salicylic acid (SA) undergoes conjugation with glycine to form the major metabolite, salicyluric acid (SUA). It is also conjugated with glucuronic acid to form phenolic (SPG) and ester or aryl (SAG) glucuronides. Ring hydroxylation of SA forms the minor metabolite, gentisic acid (GA). Further metabolites of SA have also been found, namely the phenolic glucuronide of salicyluric acid (SUA-PG) and the glycine conjugate of gentisic acid, gentisuric acid (GUA). Free SA and its metabolites are eliminated from the body by the kidney. The metabolites are subject to glomerular filtration but free SA is also actively secreted by the proximal tubule.

Pharmacokinetics

Following a therapeutic dose of 650 mg ASA, the plasma half-life of ASA is 15 minutes whereas the half-life of SA is 4 hours (see Needs and Brooks, 1985, for a review on aspirin pharmacokinetics and metabolism). Therefore, SA is distributed in and eliminated from the body more slowly than ASA. At low doses of aspirin of less than 300 mg, the overall elimination of salicylate is described by first-order kinetics (Bedford *et al.*, 1965; Levy, 1965). However, at higher, therapeutic doses some of the metabolic processes become capacity-limited as salicylate levels increase, whereas the formation of the minor metabolites, GA, SAG and the excretion of unchanged SA remain first-order (Levy *et al.*, 1972). At the

therapeutic dose of 600 mg of ASA, the maximum rate of SUA formation is approached but the formation of SPG remains a first-order process (i.e. linear). The usual therapeutic dose of ASA is 600 mg every 6 hours giving free SA plasma concentrations of less than 60 µg/ml and a plasma half-life of 4 to 6 hours. In arthritis, free SA plasma concentrations of 200 to 350 µg/ml may be required, and plasma half-life may be prolonged to 15 hours or more.

1.5 PARACETAMOL

Acetanilide was first introduced into medicine in 1886, as an effective antipyretic. However, it was found to cause cyanosis and so less toxic alternatives were sought. Baron von Mering discovered phenacetin and paracetamol, both of which are derived from acetanilide and are just as effective but less toxic. Von Mering thought paracetamol was too toxic and so phenacetin came to replace acetanilide. However, as phenacetin was found to cause severe renal necrosis, the use of paracetamol increased and in 1949, when paracetamol was recognised as the major active metabolite of both acetanilide and phenacetin, it became very popular. It is now widely used for many minor painful and febrile conditions.

a) Mode of Action and Therapeutic Uses

Analgesic Effect

The analgesic effects of aspirin and paracetamol are comparable as they are both effective in relieving pain of moderate intensity. As with aspirin, the analgesic effect of paracetamol is due to inhibition of prostaglandin synthesis. However, paracetamol is approximately 10 times less effective than aspirin as an inhibitor of peripheral prostaglandin synthetase, but it has the same potency on the prostaglandin synthetase in the brain (Ferreira and Vane, 1974). As paracetamol only has a weak anti-inflammatory action but is an effective analgesic it should be used in relieving mild to moderate pain which is not accompanied by significant inflammation, such as headache, myalgia, arthralgia and dysmenorrhea.

Antipyretic Effect

Paracetamol is effective in lowering an elevated body temperature. As with aspirin it prevents the synthesis and release of prostaglandins in the brain caused by endogenous pyrogens.

b) Toxicity

High doses of paracetamol have occasionally been found to cause destruction of erythrocytes, resulting in haemolytic anaemia. In some instances this is due to glucose-6-phosphate dehydrogenase deficiency in the red blood cell but it can also be due to paracetamol metabolites oxidising haemoglobin and glutathione and SH groups in the wall of the erythrocyte.

Paracetamol has been associated with nephrotoxicity, but due to the very large doses that have to be taken, paracetamol induced kidney damage is very rare (Prescott, 1982), although it does occur in the overdose patient.

Liver damage is the major toxic effect of paracetamol although it only occurs at high doses. It is usually associated with acute toxicity as in an overdose but there are a few instances where chronic paracetamol use has resulted in liver damage. As little as 5 g of paracetamol in a single dose can cause acute hepatic necrosis and 15 g can result in death. Liver injury is usually evident as elevation in serum bilirubin, transaminase and lactate dehydrogenase concentrations and prolonged prothrombin time. However, if the paracetamol toxicity is treated and it proves

non-fatal, the hepatic necrosis is slowly reversible over the following months.

1.6 METABOLISM OF PARACETAMOL

Absorption and Distribution

Paracetamol is rapidly absorbed from the gastrointestinal tract and peak plasma concentrations occur 30 to 60 minutes after ingestion (Heading *et al.*, 1973). Paracetamol is also subject to dose-dependent first-pass metabolism. As the dose of paracetamol increases, the proportion undergoing first-pass metabolism decreases, to around 12% at a dose of 1 g or greater (Mucklow *et al.*, 1980). The majority of the pre-systemic metabolism occurs in the liver although a small amount of paracetamol conjugation does take place in the gastro-intestinal tract (Rogers *et al.*, 1985, 1986).

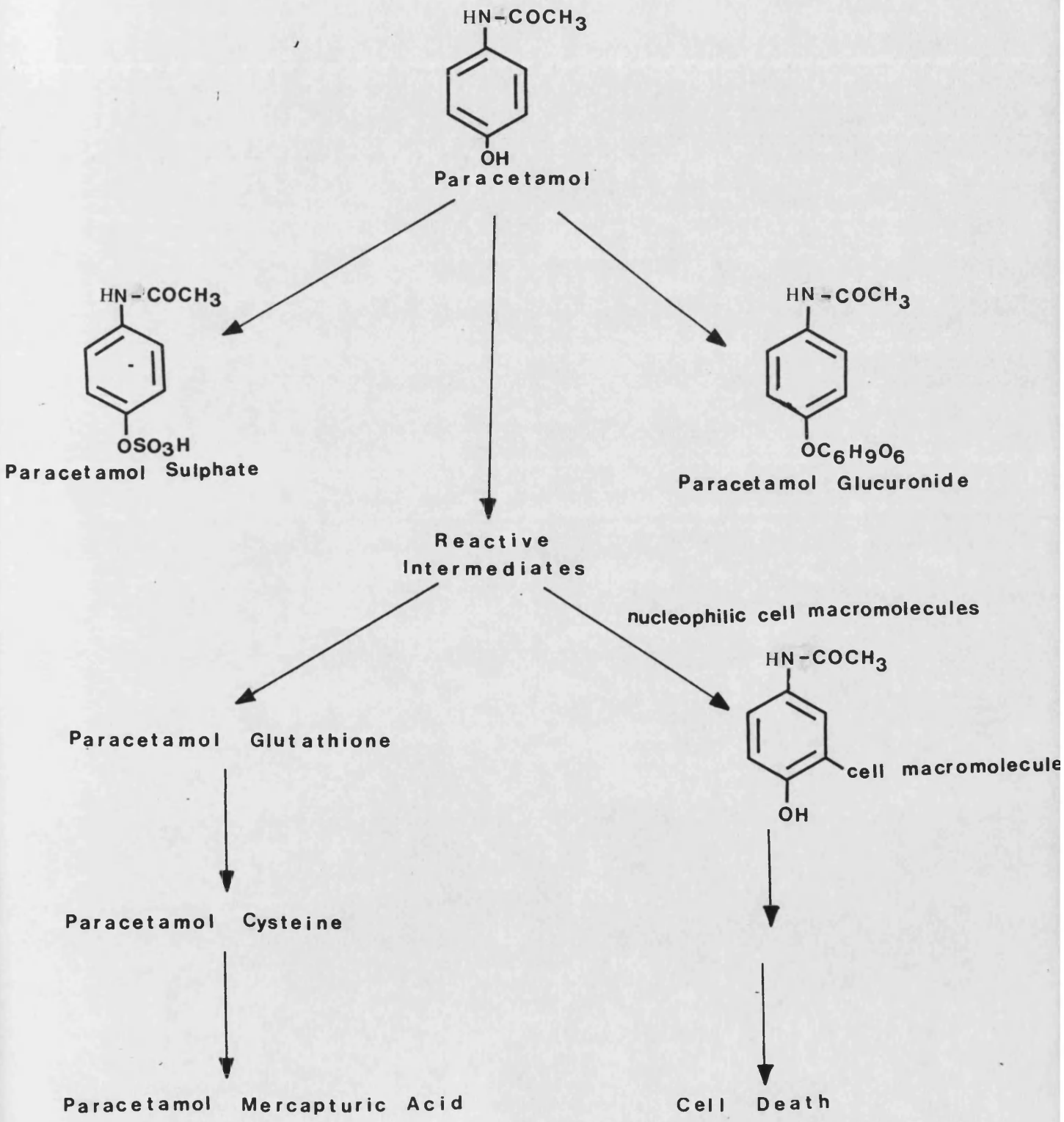
Paracetamol is distributed throughout most body fluids. At therapeutic concentrations of 60 μg/ml, paracetamol shows no plasma protein binding, but at toxic concentrations of 280 μg/ml, 15 - 21% of paracetamol is protein-bound (Gazzard *et al.*, 1973).

Metabolism

The major metabolites of paracetamol (P) are the glucuronic acid and sulphate conjugates; paracetamol glucuronide (PG) and paracetamol sulphate (PS). Paracetamol is also subject to oxidation by cytochrome P_{450} which results in the formation of a reactive intermediate. At normal therapeutic doses, this intermediate is

FIGURE 1.3

Metabolic Pathways of Paracetamol.



inactivated by further conjugation with glutathione. The glutathione conjugate is then further metabolised into paracetamol cysteine and paracetamol mercapturic acid.
Upon administration of greater doses, the larger amounts of the reactive intermediate may utilise all of the available supplies of glutathione. Therefore any excess metabolite is free to combine with cell constituents so causing liver damage.

The metabolites of paracetamol are mainly eliminated by the kidneys together with a small percentage of the unchanged parent compound.

Pharmacokinetics

Paracetamol elimination is a first-order process and it has a plasma half-life of approximately 2.5 hours (Nelson and Morioka, 1963; Rawlins *et al.*, 1977). The therapeutic dose of paracetamol is 0.5 to 1 g every 3 to 4 hours.

1.7 ORAL CONTRACEPTIVE STEROIDS

Oral contraceptive steroids (OCS) have been widely used for about 20 years and are taken by approximately 60 million women throughout the world. The most common formulation for oral contraception is the combined preparation which contains 30 to 50 μg of an estrogen (usually ethinyl estradiol) and less than 1 mg of progestogen. The combined pill is usually administered for 21 days then stopped for 7 days during which withdrawal bleeding occurs.

a) Mode of Action

The combined pill has several major sites of action including the hypothalamus and pituitary gland, the endometrium, the cervix and the Fallopian tubes.

Pituitary-hypothalamic factors

The progestogen content of the combined pill suppresses luteinizing hormone (LH) secretion by a negative feedback on the hypothalamus whilst the estrogen content suppresses follicle stimulating hormone (FSH) secretion in a similar fashion. Thus the combined pill maintains FSH and LH concentrations at normal luteal phase levels which results in the inhibition of ovulation. The lack of a follicular rise in FSH levels prevents the initiation of follicular development whilst the absence of an LH-FSH ovulatory surge prevents ovulation. Due to the suppressed LH and FSH levels and the inhibition of follicle development, endogenous estradiol concentrations in the blood remain low.

Endometrial factors

During oral contraceptive use, the endometrium is altered so that it is not receptive to ovum implantation. The progestogen component is mainly responsible for these changes whereby the endometrium regresses from a proliferative phase to a progestational phase which after several cycles of oral contraception can result in a decidualized bed with exhausted and atrophied glands. Although the endometrium is not a site for contraceptive action of the estrogenic component of the pill, it is responsible for increasing the stability of the altered endometrium, so preventing breakthrough bleeding from occurring.

Cervical factors

The progestogen component has a further contraceptive effect on the cervical mucus resulting in hindered sperm movement. It remains thick as in the normal luteal phase and is impervious to sperm transport.

Fallopian tube factors

The motility of Fallopian tubes and hence ovum movement is altered by the progestogen component of the combined pill, which may be a further contraceptive effect.

b) Toxicity

The combined oral contraceptive steroids (OCS) have been associated with many metabolic and systemic effects. A review of the side effects of OCS is given by Odell and Molitch, 1974.

Thromboembolism

One of the undesired effects that is associated with oral contraceptive steroids is the increased risk of deep venous thrombosis. OCS have been found to increase the relative risk of thromboembolism by a factor of 11 (Boston Collaborative Drug Surveillance Program, 1973). Further documentation links the increased risk of thrombosis with the dose of estrogen - as a result of which the dose of ethinyl estradiol present in the pill has been reduced from 100 µg or more/day to 30 - 35 µg/day. However, even at the lower doses of ethinyl estradiol, the increased risk of thrombosis is still present as confirmed by Stolley *et al.*, 1975.

The mechanism by which oral contraceptives cause vascular complications is not known with certainty, however it is known that OCS alter blood coagulability. Oral contraceptive use has been found to elevate plasma levels of fibrinogen and factors VII and X whereas antithrombin III levels were found to decrease (Dugdale *et al.*, 1971; Meade *et al.*, 1976). All of these changes favour thrombosis but they are largely offset by an increase in fibrinolytic activity.

Hypertension

There is a small risk of developing hypertension or exacerbating existing hypertension whilst using OCS (Weir *et al.*, 1971 and Karagh *et al.*, 1967). The estrogenic component is responsible for the increased incidence of hypertension and it is thought to activate the renin-angiotensin system. However, unlike

thromboembolism, the relative risk of hypertension is not decreased with a decrease in the dose of estrogen.

Carcinogenicity

Steroid contraception has been feared to cause cervical and breast cancer. However, there is no evidence to date to indicate that OCS increase the incidence of cancer. In fact, OCS have been found to have a protective effect towards benign breast tumours (Royal College of General Practitioners, 1974).

Glucose Tolerance

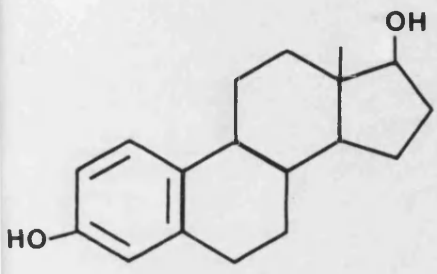
Glucose tolerance is impaired in 15 to 40% of OCS users and it is associated with elevated plasma concentrations of insulin and blood sugar. This effect is caused by an increase in peripheral resistance to insulin action but its clinical significance is uncertain.

Lipid Metabolism

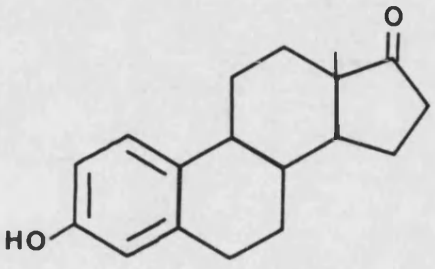
Oral contraceptives have been found to increase serum triglycerides, phospholipids and cholesterol concentrations, with the estrogenic component being responsible for the elevation of the first two only (Wynn *et al.*, 1966; Stokes and Wynn, 1971). As elevated serum lipid and lipoprotein concentrations are associated with atherosclerosis, OCS may accelerate atherosclerosis in susceptible women, i.e. with either a family history or elevated serum lipids.

1.8 METABOLISM OF ENDOGENOUS ESTRADIOL

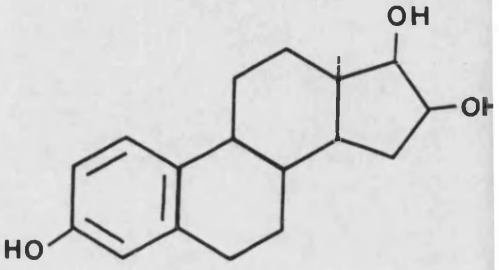
There are 3 naturally occurring estrogenic hormones, namely estradiol (E_2), estrone (E_1) and estriol (E_3). Of these estradiol is the most potent and estriol has the weakest activity. The structures of these hormones are given below.



Estradiol



Estrone



Estriol

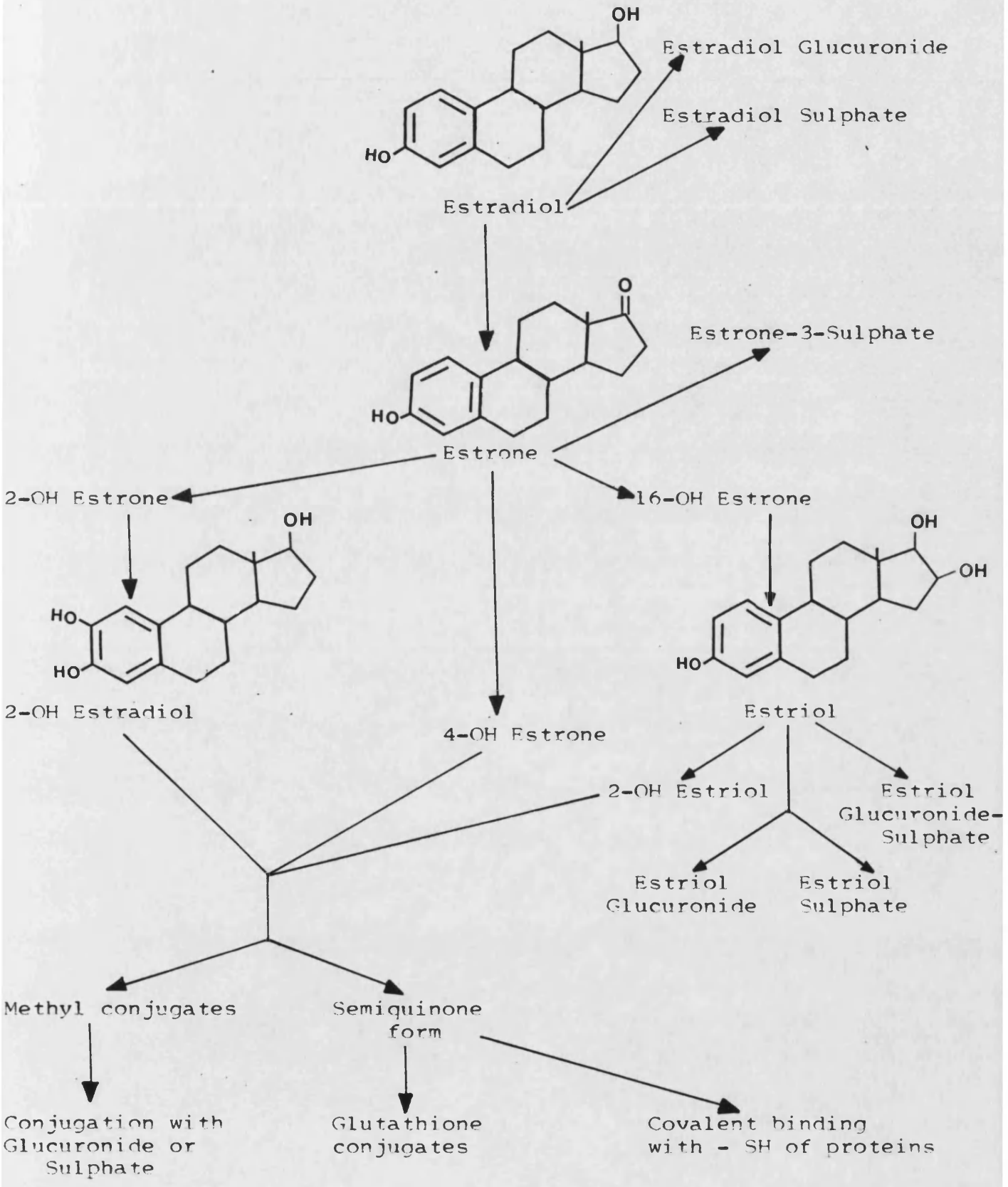
Upon administration of exogenous estradiol, 50 to 80% of the dose is excreted in the urine within 4 to 6 days, mainly as glucuronide conjugates and up to 20% is recovered in the faeces (Sandberg & Slaunwhite, 1957; Beer and Gallagher, 1955).

Phase I metabolism of estradiol

Estradiol is converted into estrone by 17β hydroxysteroid-dehydrogenase and it is estrone which appears to be the precursor of other estrogen metabolites. Estrone can then undergo further oxidation at C-16 by cytochrome P_{450} to form estriol. Hydroxylation of estrogens can occur at C-2, C-4, C-6, C-7, C-11, C-14, C-15, C-16 and C-18 but the 2 major pathways involve C-2 and C-16. Hydroxylation at C-16 produces estriol whilst hydroxylation at C-2 (and C-4) produces the catechol estrogens. 2-hydroxylation and 16β-hydroxylation are competitive in nature as an increase in 16β -hydroxylase activity (as caused by an increase in body weight

FIGURE 1.4

Major metabolic pathways of estradiol



or cirrhosis of the liver or lowered level of thyroid hormones) results in a decrease in 2-hydroxylation (Fotherby, 1984).

Phase II metabolism of estradiol

Glucuronidation and sulfation are the major conjugation pathways of estrogens and formation of these metabolites usually results in rapid excretion from the body via urine or bile. Glucuronidation has been found to favour particular sites of the estrogen molecule, namely C-3 and the D-ring and as such estriol glucuronides are the major urinary metabolites of the estrogens but glucuronidation of estradiol and estrone also occurs. Formation of estriol glucuronides occurs mainly in the liver, whereas estradiol and estrone glucuronides are thought to be formed at extrahepatic sites such as the kidney and the intestine (Hobkirk and Nilsen, 1974). Glucuronidation appears to be the major form in which estrogens are excreted in the urine but sulphation of the estrogens also occurs. Of these, estrone 3 sulphate is the most important, as this has been found to be the major circulating estrogen, with its plasma concentration exceeding that of estradiol about 10-fold (Longcope & Williams, 1974). Estrone 3 sulfate is rapidly formed from estrone in the liver and it is reconverted to estrone and estradiol by sulfatases and 17-β-hydroxysteroid dehydrogenase found in the liver and also in target tissues such as the uterus. Estrone 3 sulfate is regarded as a storage form of estradiol/estrone being in equilibrium with the free and active estrogens (Fotherby, 1984). Sulfation of estrone and estradiol occurs primarily in the liver but it may also occur at extrahepatic sites such as the intestine.

Double conjugates of estrogens have also been reported where glucuronide and sulphate conjugation occurs at 2 separate hydroxyl groups.

Catechol estrogens can be excreted in the urine unchanged but the majority undergo further transformation. The most important metabolic reaction for these estrogens is methylation catalysed by catechol-O-methyl transferase. The methoxy catechol estrogens produced by methylation then undergo further conjugation with glucuronide or sulphate prior to urinary excretion. A small percentage of the catechol estrogens are oxidised into the semiquinone form prior to either glutathione conjugation and urinary excretion or covalent binding to the -SH group of proteins.

Enterohepatic circulation

As well as the transformation of estrogens and/or their conjugation and excretion into the urine, these steroids are also further inactivated by biliary excretion. Following exogenous administration of estradiol, 1 to 18% of the dose appears in the faeces whilst approximately 50% of the dose is excreted in bile, thus indicating that re-absorption of the estrogens has occurred (Sandberg and Slaunwhite, 1957).

Estradiol is excreted into the bile mainly as the glucuronide, estrone mainly as the sulphate and estriol mainly as the double conjugate. These conjugates are then hydrolysed in the intestinal tract and largely reabsorbed in the unconjugated form although some glucuronidation or sulphation may occur in the mucosal cells during absorption (Adlercreutz, 1970).

This circulation of estrogens results in their excretion from the body being delayed. However, as the reabsorption from the intestine is not complete, up to 20% of estrogens are excreted in the faeces.

Protein binding

Estradiol, estrone and estrone sulphate all bind to plasma proteins. Estradiol binds to albumin and the sex hormone binding globulin (SHBG) whereas estrone and estrone sulphate bind to albumin alone (Westphal, 1971).

1.9 METABOLISM OF ETHINYL ESTRADIOL

Natural estrogens are relatively inactive when given orally in comparison to synthetic estrogens. For example, whereas 30 - 50 µg of ethinyl estradiol is administered per day in OCS, 4 mg of estradiol would have to be administered to achieve a comparable effect. This is probably due to the rapid clearance of endogenous hormones from the blood and an extensive first-pass effect. However, in order for synthetic estrogens to gain estrogenic properties, they share structural properties with the natural endogenous estrogens and therefore they may be metabolised by the same enzymes which metabolise the natural estrogens. As ethinyl estradiol (EE_2) is orally active, it has a slower elimination from the circulation if compared to estradiol. Like estradiol, urinary excretion of mainly glucuronide conjugates occurs, together with extensive metabolism at the steroid nucleus. Also, similar amounts of estradiol and ethinyl estradiol are excreted in the bile.

However unlike estradiol, ethinyl estradiol metabolites do not leave the body mainly via the urine. Urinary recovery is much lower than that of estradiol, whilst faecal recovery is much increased, resulting in a urine/faecal ratio of about 6 : 4 for ethinyl estradiol compared to the ratio of 8 : 2 for estradiol.

Phase I metabolism

As with the natural estrogens, the major pathway for ethinyl estradiol is aromatic hydroxylation with 2-hydroxy ethinylestradiol being the major metabolite (accounting for 29 to 64% of the dose) although 4-hydroxylation may occur. Further hydroxylation of EE_2 at C-16 and C-6 have also been found.

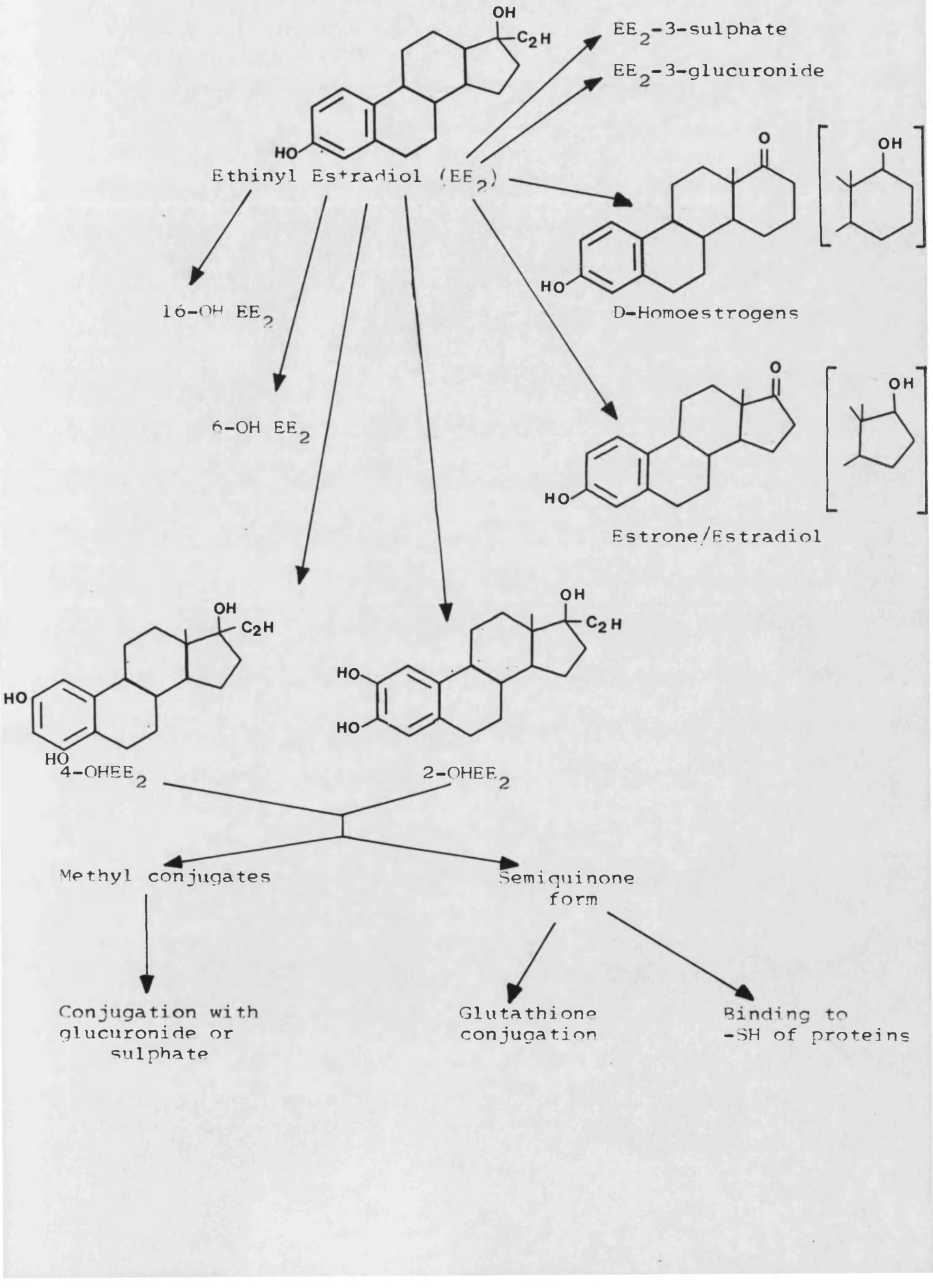
Unlike estradiol, no hydroxylation at C-16 occurs which leads to the formation of estriol. This is mainly due to estrone being the substrate for estrogen 16β-hydroxylase and the introduction of the ethinyl group at C-17 prevents the estrone analogue from being formed. The lack of 16 β hydroxylation and subsequent estriol formation is thought to be the reason for the slower breakdown of ethinyl estradiol.

De-ethinylation of ethinyl estradiol has been found resulting in the formation of estradiol and estrone although controversy exists concerning the quantitative extent. 15 to 20% of the total glucuronide metabolites of EE_2 in urine were found to be de- ethylated, whereas only 1 - 2% of mestranol was found to undergo de-ethylation, even though 50% of mestranol is converted into EE_2.

D-homoannulation is a minor metabolic pathway of ethinyl estradiol that is not found in estradiol metabolism. It involves enlargement

FIGURE 1.5

The major metabolic pathways of ethinyl estradiol



of the D ring and oxidative elimination of an ethinyl carbon atom.

Phase II metabolism

Ethinylestradiol can be directly conjugated to form EE_2-3-glucuronide and EE_2-3-sulfate. The glucuronide conjugate is excreted in the urine but it is also subject to enterohepatic circulation. The sulfate conjugate is the principal circulating form of EE_2 with its plasma levels approximately 10 times higher than those of EE_2. Therefore it is considered to play the same role as estrone-3-sulfate whereby it represents a plasma transport and storage form of EE_2 from which hydrolytic enzymes may release free and active EE_2.

The products of aromatic hydroxylation of EE_2 (2-hydroxy and 4-hydroxy ethinyl estradiol) undergo the same metabolic pathways as utilised by the catechol estrogens as discussed in 1.8. That is, they are subject to conjugation with glutathione, covalent binding to proteins and methylation with catechol-O-methyl transferase. The major urinary metabolite of EE_2 is 2-methoxy EE_2 which is excreted as the sulfate or the glucuronide conjugate (see Helton & Goldzieher, 1977 and Ranney, 1977, for reviewson ethinyl estradiol metabolism and pharmacokinetics).

Pharmacokinetics of ethinyl estradiol

Ethinyl estradiol is rapidly and completely absorbed from the intestine but large interindividual variations in the plasma levels and the pharmacokinetics of EE_2 have been reported.

Plasma levels of EE_2 are known to decline bi-exponentially with the

initial decline having a half-life of about 1 hour whilst the second decline has a half-life of 7 to 8 hours (Warren and Fotherby, 1973). However, Back *et al.*, (1979) described the occurrence of a secondary plasma peak at around 12 hours following the oral administration of 50 µg EE_2, with a bi-exponential decline before this time point. This delayed secondary peak is indicative of extensive enterohepatic circulation of EE_2, as found with estradiol. The terminal decline has been found to have a half-life of 9.5 to 18.5 hours (Back *et al.*, 1980).

The pharmacokinetics of ethinyl estradiol have been found to vary greatly between ethnic groups. Nigerian women have a shorter half-life of EE_2 than South and South-East Asian women, with American women having the longest half-life of all 3 groups (Helton and Goldzieher, 1977; Williams *et al.*, 1980). Differences in metabolism between these races have also been found. Nigerian women demonstrated relatively little oxidation and diglucuronides predominated in the urine whilst American women demonstrated a high degree of oxidation and monoglucuronides predominated, with South Asian women holding an intermediate position in oxidation and glucuronidation (Williams and Goldzieher, 1980).

1.10 SCOPE OF THE THESIS

Genetic and environmental control of phase I metabolism has been extensively studied. However, until recently little was known about the effects of these factors on phase II metabolism. Therefore, the effects of both genetic and environmental factors on phase II metabolism were examined in the present work, as phase II metabolism is generally responsible for the inactivation of a compound (exogenous and endogenous) NOT phase I metabolism, as shown in Figure 1.6. Aspirin and paracetamol are both commonly used drugs which are mainly inactivated by phase II metabolism and so they are suitable probe drugs to investigate genetic and/or environmental control of conjugation.

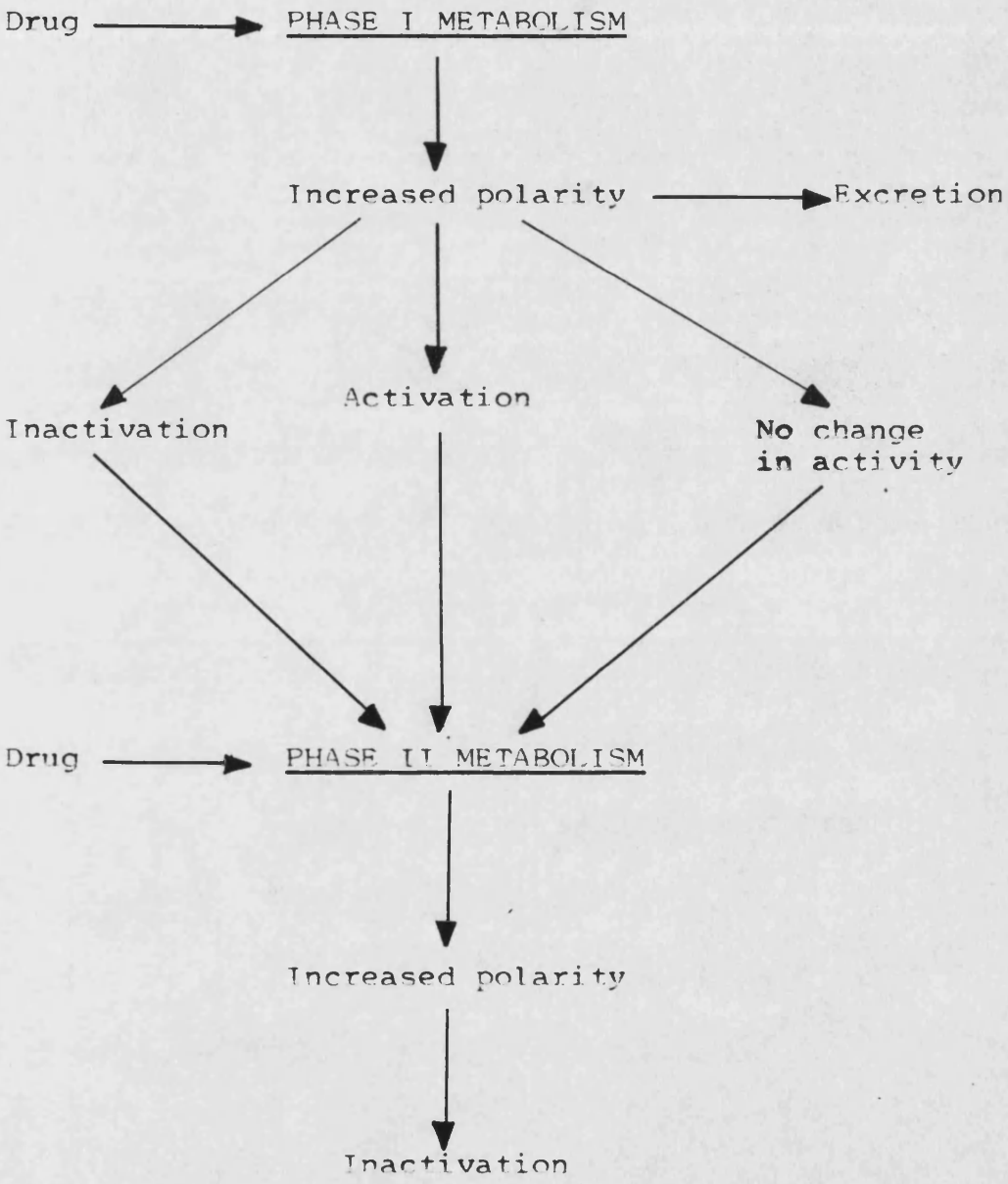
There are wide interindividual variations in the plasma concentrations of aspirin and paracetamol, when a standard therapeutic dose is given to subjects for the control of pain in conditions such as rheumatoid arthritis. Such variation indicates that one or more factors are affecting the concentrations of salicylic acid or paracetamol. One major factor responsible for the concentration of a drug is drug metabolism, and interindividual variations in the metabolism of aspirin and paracetamol have been reported.

The population studies of aspirin and paracetamol, using healthy volunteers of a wide age-range, were undertaken to determine the factor(s) (both genetic and environmental) responsible for the variation in metabolism.

The population studies of aspirin and paracetamol can also determine the effects of the environmental factor, the use of oral

FIGURE 1.6

Summary of phase I and phase II metabolism.

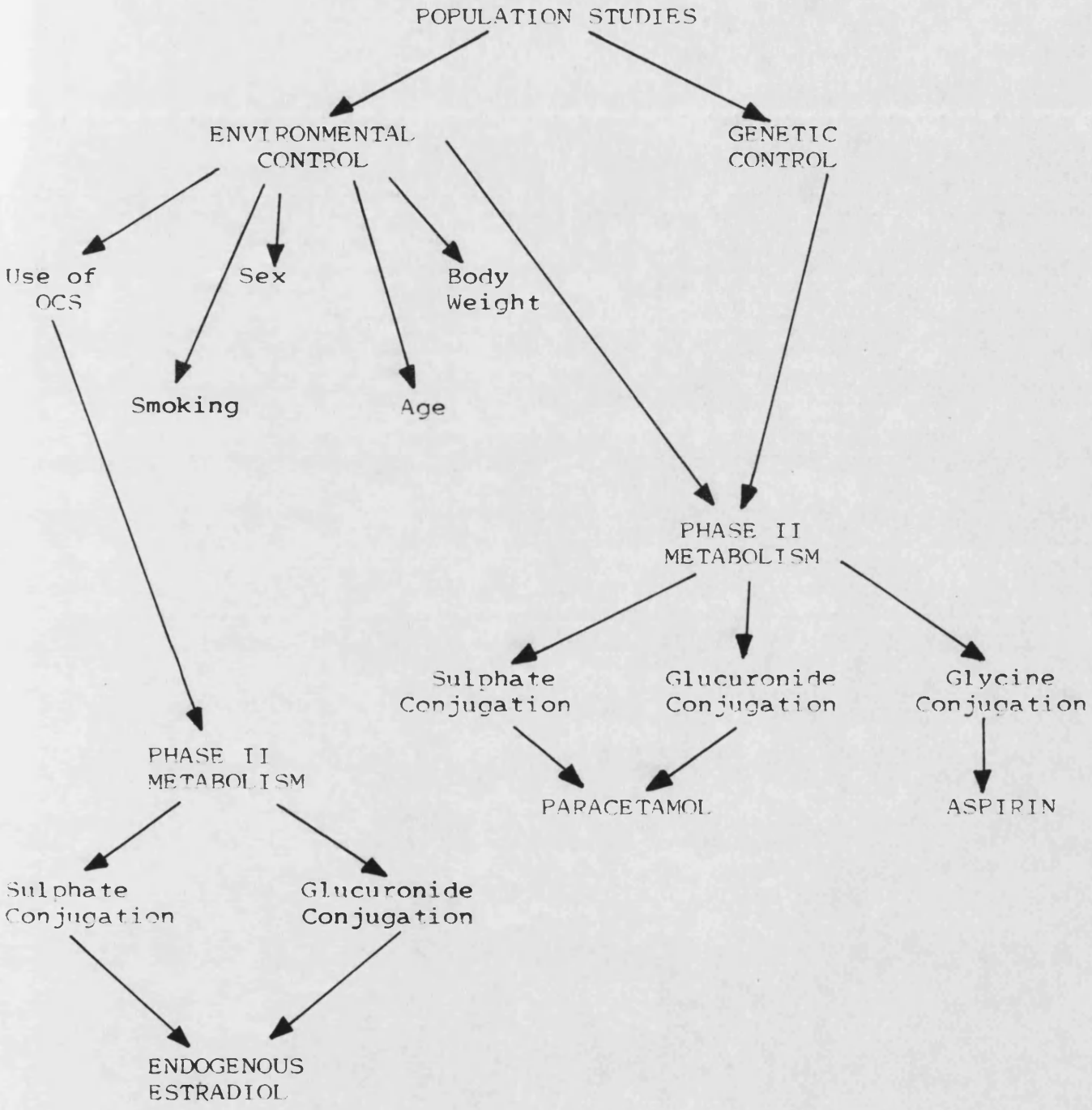


contraceptive steroids (OCS), on the glycine, glucuronide and sulphate conjugation of these exogenous, foreign compounds. However, little is known about the effect of OCS on endogenous metabolism. Therefore a population study was performed in healthy female volunteers to determine the effects of chronic OCS use on the metabolism of the endogenous hormone estradiol. As with the exogenous compounds, the effect on the major phase II metabolic reactions; glucuronide and sulphate conjugation were studied. Figure 1.7 depicts the factors investigated in the human studies performed in this work.

Rat studies were also performed to determine the effect of chronic ethinyl estradiol administration, the usual estrogenic component of OCS, on the metabolism of the exogenous compound, paracetamol and the endogenous compound, estradiol. All 3 compounds are subject to glucuronide and sulphate conjugation and so this study can determine if there is any direct competition in the metabolism of these compounds. It can also determine if the estrogenic component of OCS is responsible for the effects of oral contraception on drug metabolism.

FIGURE 1.7

Diagrammatic representation of the factors investigated in the human studies.



CHAPTER TWO

MATERIALS AND METHODS

2.1 COMPOUNDS

a) Drugs

i) soluble aspirin BP tablets, each tablet contained 300 mg. (Pharmacy, Royal United Hospital, Bath).

ii) ^{14}C-17β -estradiol - specific activity = 57.5 mCi/mmol (estra-1,3,5(10)triene-3,17β -diol) MW = 272.4 (Du Pont Chemicals Ltd.)

iii) 17β-ethinyl estradiol (17β ethinyl-1,3,5(10)estriene-3,17β diol) MW = 296.4 (Sigma Chemical Co.).

iv) hypnorm - Fentanyl citrate (0.315 mg/ml) + Fluanisone (10 mg/ml) (Janssen Veterinary Products Ltd.).

midazolam hydrochloride (5 mg/ml) (Roche Products Ltd.)

The anaesthetic was prepared by adding 1 ml of hypnorm to 2 ml of 0.9% saline followed by 1 ml of midazolam.

v) paracetamol B.P. tablets, each tablet contained 500 mg (Pharmacy, Royal United Hospital, Bath).

b) Analytes

i) o-anisic acid (o-methoxybenzoic acid) $O\text{-}CH_3OC_6H_4CO_2H$. MW = 152.15 (Aldrich Chemical Co. Ltd.)

ii) gentisic acid (2,5-dihydroxybenzoic acid)-sodium salt $(OH)_2C_6H_3COONa$, MW = 176.1 (Sigma Chemical Co.)

iii) paracetamol B.P. (4-acetominophenol) $CH_3CONHC_6H_4OH$ MW = 151 (Pharmacy, Royal United Hospital, Bath).

iv) paracetamol glucuronide (4-hydroxy N-phenyl acetamide 4-glucuronide) p-$CH_3CONHC_6H_4OC_6H_9O_6$, MW = 327 (kindly donated by Stirling Winthrop, R & D).

v) paracetamol sulphate (4-hydroxy N phenylacetamide-4-sulphate), p-$CH_3CONHC_6H_4OSO_3H$, MW = 230 (kindly donated by Stirling Winthrop, R & D).

vi) salicylic acid (2-hydroxybenzoic acid)-sodium salt HOC_6H_4COONa, MW = 160.1 (Sigma Chemical Co.)

vii) salicyluric acid (o-hydroxyhippuric acid) $HOC_6H_4CONHCH_2CO_2H$, MW = 195.2 (Sigma Chemical Co.)

c) Solvents

i) acetone - analytical grade, CH_3COCH_3, MW = 58.08 (Fisons)

ii) acetonitrile (methyl cyanide) - HPLC grade. CH_3CN, MW = 41.05 (Fisons)

iii) chloroform - analytical grade, $CHCl_3$, MW = 119.4 (Fisons)

iv) isopropanol - HPLC grade, $(CH_3)_2CHOH$, MW = 60.1 (Sigma Chemical Co.)

d) Other Chemicals

i) formic acid, 98% HCOOH, MW = 46.03 (BDH Chemicals Ltd.)

ii) glucurase enzyme - (β-D-Glucuronide glucurono-

hydrolase from bovine liver). Buffered at pH 5.0, 5000 units/ml (Sigma Chemical Co.)

iii) hydrochloric acid, HCl, MW = 36.46, SLR Grade (Fisons)

iv) perchloric acid, 60% $HClO_4$, MW = 100.46 (Fisons)

v) potassium dihydrogen orthophosphate, KH_2PO_4, MW = 136.09 (Fisons)

vi) orthophosphoric acid, 88%, H_3PO_4, MW = 98 (Fisons)

vii) saccharic acid 1,4 lactone, MW = 192.1 (Sigma Chemical Co.)

viii) sodium hydroxide, NaOH, MW = 40.0, AnalaR (BDH Chemicals)

ix) sulfatase enzyme (aryl sulfate sulfohydrolase from helix pomatia - type H5) 19800 units/g with β-glucuronidase activity (Sigma Chemical Co.).

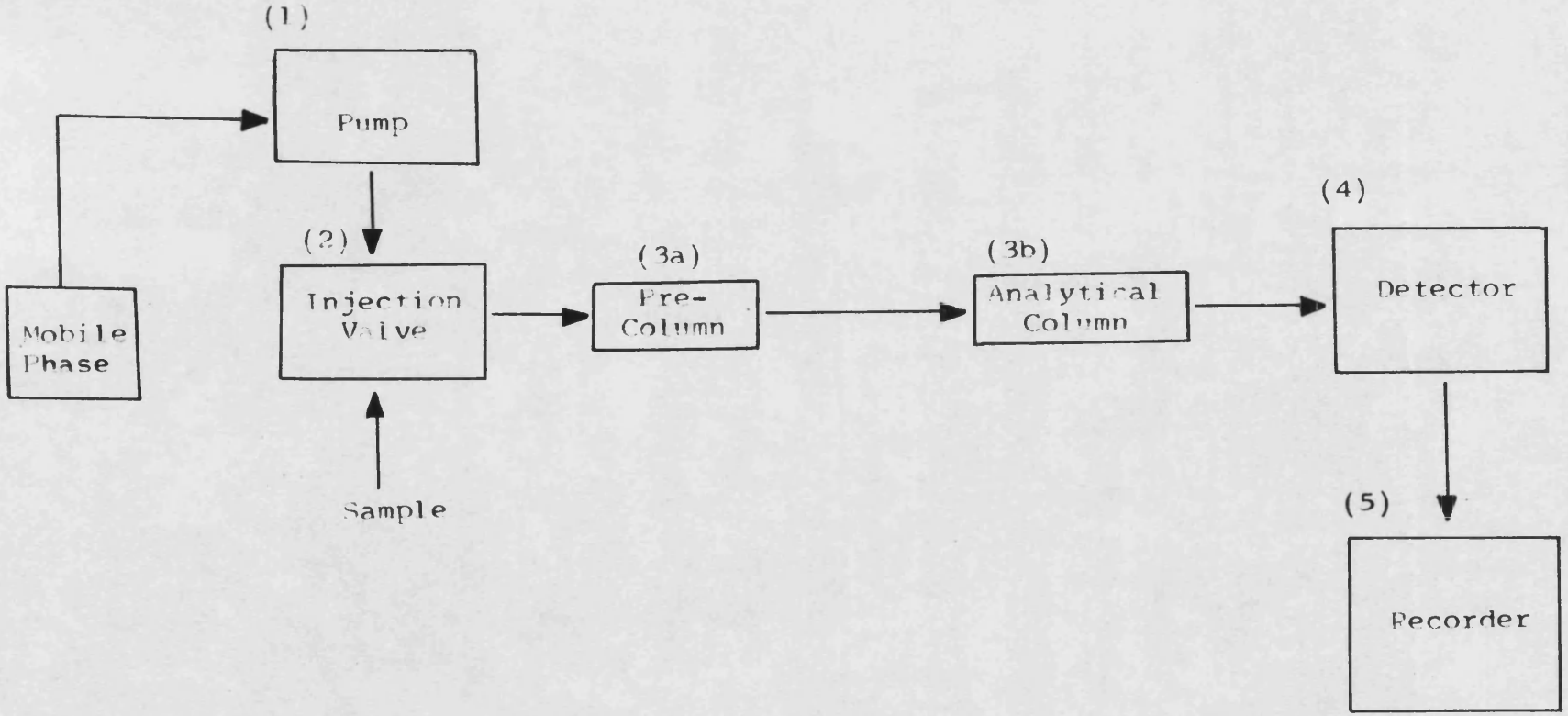
2.2 INSTRUMENTATION

a) High Performance Liquid Chromatography (HPLC)

Fig. 2.1 shows a generalised diagram of the HPLC system used. The mobile phase was pumped to the columns at a constant flow rate by a Laboratory Data Control (LDC) Constametric III Metering Pump (1) which provides a pulsation less flow using dual-reciprocating pistons. Samples were injected into the system using a rheodyne injection valve (2) fitted with a 20 µl loop. Eluted compounds were detected on an LDC spectromonitor III Spectrophotometer (4) with a wavelength range in the UV spectrum of 190 to 350 nm. (All of the

FIGURE 2.1

Generalised diagram of an HPLC system.



above equipment was purchased from LDC, Stone, Staffs). Chromatographs were produced on a JJ Model CR550 potentiometric chart recorder (5) (JJ Lloyd Instruments Ltd.).

Both the 5 cm long precolumn (3a) and the 25 cm long analytical column (3b) consisted of stainless steel tubes with a 5 mm internal diameter, ¼" external diameter and were packed with reversed-phase microparticle silica, 5 µm (Hypersil ODS, Shandon Products Ltd.). The column end fitting (female) consisted of a low dead-volume Swagelok connection with a 1/16" outlet which was connected to the detector by microbore Teflon tubing (internal diameter, 0.15 mm). Low dead-volume 1/16" stainless steel tubing (internal diameter, 0.15 mm) was used to connect the injection valve to the top of the column. All other connections were made of stainless steel tubing and standard Swagelok fittings.

The equipment was operated at ambient temperature throughout.

Mobile phase was degassed by sparging with helium for 5 minutes prior to use.

b) Liquid Scintillation Counter

Radioactivity was measured in a LKB-Wallac Model 1215 Rack Beta Liquid Scintillation Counter. This automatic, microcomputer controlled four channel scintillation counter is ideal for counting

beta emissions over the energy range 1 kev to 2800 kev. The samples were counted with external standard channels ratio method (ESCR).

2.3 HUMAN VOLUNTEERS

All human studies received approval from the Bath Health District Research Ethics Committee. All volunteers were judged healthy following an interview with a consultant physician and gave their informed consent.

For the aspirin and paracetamol studies, volunteers of both sexes aged between 18 and 65 years were recruited from the Pharmacology Group, University of Bath; the Royal United Hospital, and the Ministry of Defence sites, Foxhill and Ensleigh.

For the estradiol study, female volunteers aged between 18 and 35 years were recruited from the Pharmacology Group and the Family Planning centres both at the University of Bath and at the Sawclose Family Planning Clinic, Bath. Two groups of young premenopausal women were recruited. One group had taken oral contraceptive steroids (OCS) for a minimum of 6 months whilst the second control group had never taken OCS. Details of oral contraceptive use and the day of the menstrual cycle on which the study was performed were recorded.

For all studies a record was made of the age and weight of the volunteers together with all drugs currently being taken.

2.4 ANIMALS

The experimental animals used were female Wistar rats (Bath University strain) weighing between 200 - 220 g.

Prior to collection of biological samples the rats were housed in groups of 10 per cage (NKP cages). Food pellets (Labsure diet CRM) and water were allowed *ad libertum* and a constant light-dark cycle was maintained (12 h light 7.00 to 19.00, 12 h dark 19.00 to 7.00) at an ambient temperature of 24°C.

To allow separate collection of urine and faeces the animals were placed in individual metabolism cages (NKP cages MCI) for 24 hours. Water was allowed *ad libertum* but food was restricted whilst urine was collected. Other conditions remained unaltered.

2.5 ADMINISTRATION OF DRUGS

a) To Humans

Each subject took soluble aspirin, 600 mg (2 x 300 mg tablets) in 50 ml water or paracetamol, 1 g (2 x 0.5 g tablets) at approximately 8 am after voiding the bladder. All urine passed fron 0 to 8 hours was collected in a urine container with no preservative.

For the estradiol study no drugs were administered. However, during a specific interval of the menstrual cycle a single 10 ml venous blood sample was obtained and urine was collected for 24 hours in a urine container with no preservative. For all studies urine volume was recorded, together with urine pH for the aspirin study, and 2 x

10 ml aliquots taken. The blood was placed in heparinized tubes and then centrifuged at 3000 rpm for 15 min to obtain the plasma.

b) To Animals

For the paracetamol study the rats were treated orally for 40 days, between 9 and 11 am, with 1 ml of either 5% ethanol or ethinyl-estradiol in 5% ethanol (11 μg/ml), the 2 groups being randomly allocated. On day 29 of treatment each animal received a single oral dose of paracetamol (50 mg/kg) and was then placed in a metabolism cage and urine collected from 0 to 8 and 8 to 24 hours. On day 40 a second oral dose of paracetamol (50 mg/kg) was administered; the animals were anaesthetised with hypnorm and medazolam (0.1 ml/25 g) and then bled by cardiac puncture at intervals over 8 hours. Each animal was bled up to 4 times, with a maximum of 2 ml of blood taken at each bleed.

For the estradiol study the rats were again treated for 40 days as above. On day 40 half of the rats were anaesthetised with hypnorm and medazolam (0.1 ml/25 g) and the jugular vein and carotid artery were cannulated. ^{14}C-17β estradiol (26.6 μg E_2; 5.075 μCi/kg) was administered through the jugular vein and blood was removed from the carotid artery at intervals over 3 hours. Each animal was bled up to 8 times with a maximum of 0.5 ml of blood taken at each bleeding. The remaining rats had ^{14}C-estradiol (26.6 μg estradiol; 5.07 μCi/kg) injected iv into the tail vein on day 40 and were then placed in metabolism cages for a 24-hour urine and faeces collection. The animal was then sacrificed and the kidney, liver, small

and large intestine removed.

In both animal studies the blood was allowed to clot in serum tubes before being centrifuged at 3000 rpm for 15 minutes.

2.6 STORAGE OF BIOLOGICAL SAMPLES

All samples were stored at -20°C prior to analysis. Urine was stored in approximately 2 x 10 ml aliquots where possible. Serum samples were stored in unheparinized tubes, plasma samples in heparinized glass tubes.

2.7 ANALYSIS OF ASPIRIN METABOLITES

Salicylic acid (SA) and salicyluric acid (SUA) were assayed in human urine by HPLC. The method was a modification of that of Cham *et al.* (1980), using o-anisic acid as the internal standard.

HPLC Conditions

Mobile phase :	30% acetonitrile
	70% orthophosphoric acid (0.1%)
	pH = 2·45
Flow rate :	1.5 ml/min
Detection :	298 nm
Chart speed :	0.4 cm/min

Assay Procedure

1 ml of urine was added to 1 ml of internal standard (o-anisic

acid, 0.5 mg/ml in distilled water). 100 µl was injected onto the column.

Analyte	Retention Time	λ_{max} (nm)
salicyluric acid	3 min 55 sec	237, 297
o-anisic acid	4 min 47 sec	235, 292
salicylic acid	7 min 33 sec	230, 295

A typical chromatogram of SA and SUA in human urine following an oral dose of aspirin is given in Fig. 2.2.
The calibration curves for SA and SUA were found to be linear over the ranges 6 to 120 µg/ml and 50 to 1008 µg/ml respectively, as shown in Fig. 2.3.

2.8 ANALYSIS OF PARACETAMOL AND ITS MAJOR METABOLITES

a) In Urine

Paracetamol (P), paracetamol glucuronide (PG) and paracetamol sulphate (PS) were assayed in human and rat urine by HPLC. The method was a modification of that of Adriaenssens and Prescott (1978) using gentisic acid as the internal standard.

HPLC Conditions

Mobile phase : 97.5% 0.08 M KH_2PO_4
2.5% isopropanol
0.1% formic acid
pH = 3.46

FIGURE 2.2

Typical chromatogram for salicylic acid (SA) and salicyluric acid (SUA) in human urine

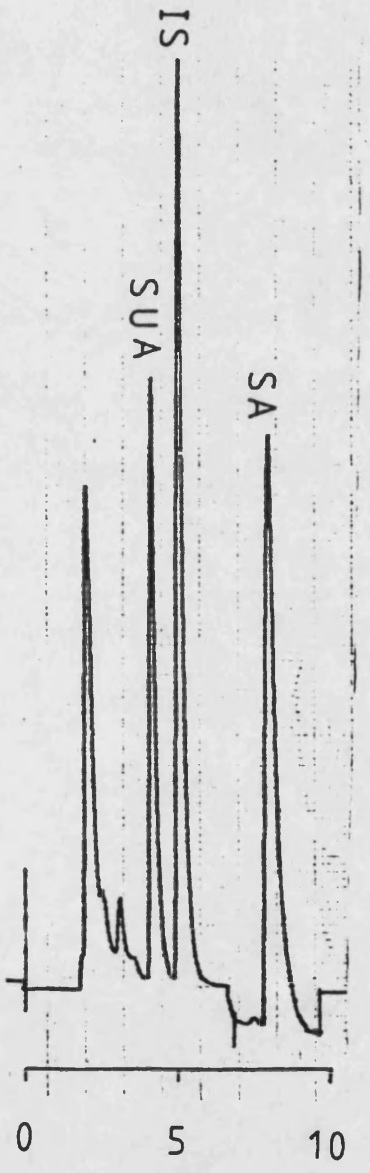


FIGURE 2.3a

Calibration curve for salicyluric acid (SUA) in human urine

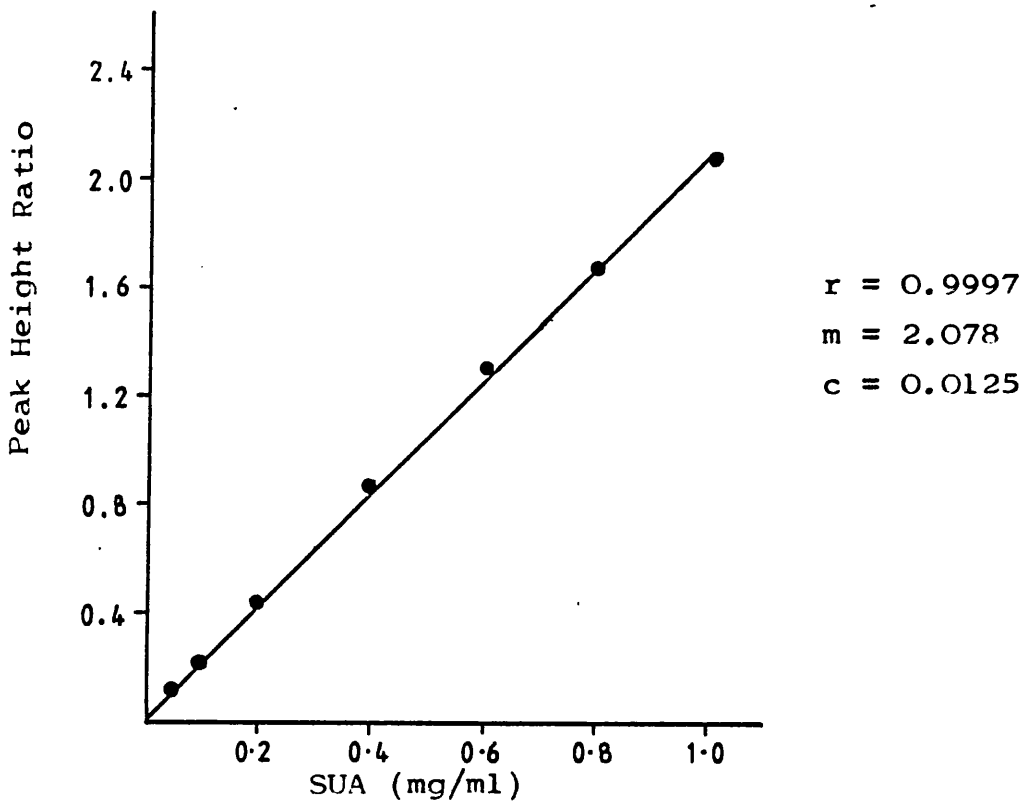
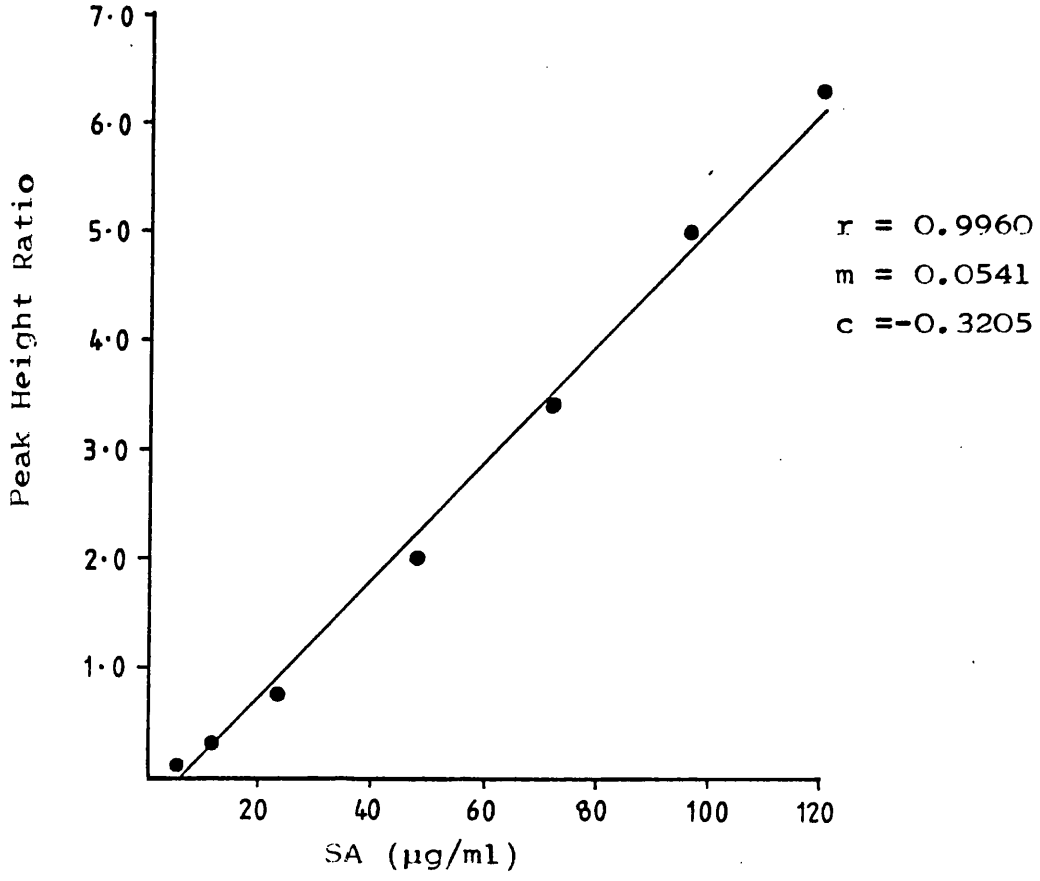


FIGURE 2.3b

Calibration curve for salicylic acid (SA) in human urine



Flow rate :	1.5 ml/min
Detection :	254 nm
Chart speed :	0.4 cm/min

Assay Procedure

1 ml of urine was added to 1 ml of internal standard (gentisic acid, 5.0 mg/ml in distilled water) and made up to 10 ml with distilled water. 100 µl was injected onto the column.

Analyte	Retention Time	λ_{max} (nm)
Paracetamol glucuronide	3 min 47 sec	242
Paracetamol sulphate	6 min 12 sec	240
Paracetamol	8 min 15 sec	242
Gentisic acid	10 min 14 sec	243

A typical chromatogram of P, PG and PS in human urine following an oral dose of paracetamol is given in Fig. 2.4(a).
The calibration curves for P, PG and PS were found to be linear over the ranges 9.6 to 192.0 µg/ml, 161 to 3220 µg/ml and 74 to 1482 µg/ml respectively. Typical calibration curves for paracetamol metabolites in urine are shown in Fig. 2.5.

b) In Serum

Paracetamol in serum was assayed in rat serum by HPLC using the modification of the method by Adriaenssens and Prescott (1978) as given above.

FIGURE 2.4a

Typical chromatogram for paracetamol and its metabolites in human urine.

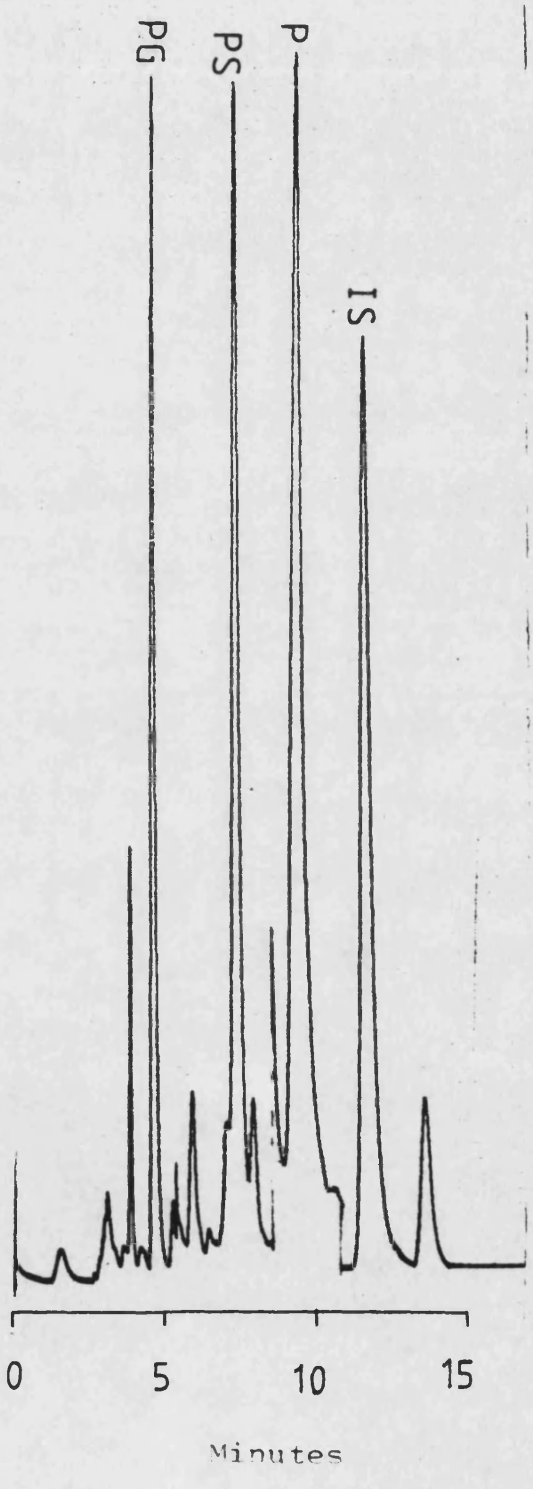
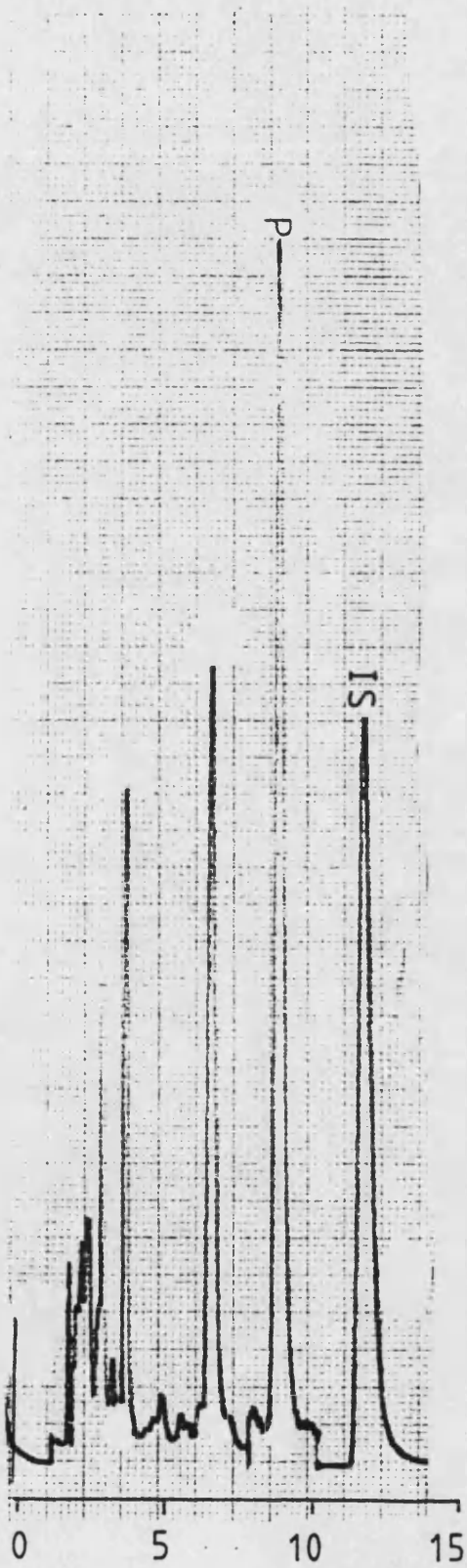


FIGURE 2. 4b

Typical chromatogram for paracetamol in rat serum.



HPLC Conditions

See 2.8(a).

Assay Procedure

0.5 ml of serum was added to 0.5 ml of internal standard (5.0 mg/ml gentisic acid). A further 0.5 ml of perchloric acid (15%) was added and after mixing, centrifuged at 3000 rpm for 15 minutes. 100 µl of the supernatant was injected onto the column.

Analyte	Retention Time	λ_{max} (nm)
Paracetamol	9 min 56 sec	242
Gentisic acid	13 min 17 sec	243

A typical chromatogram of paracetamol in rat serum following an oral dose of paracetamol is given in Fig. 2.4(b).
The calibration curves for paracetamol in serum were linear over the ranges 0.55 to 5.5 µg/ml and 3.15 to 63.0 µg/ml as shown in Fig. 2.6.

2.9 ANALYSIS OF 17β-ESTRADIOL

a) In Plasma

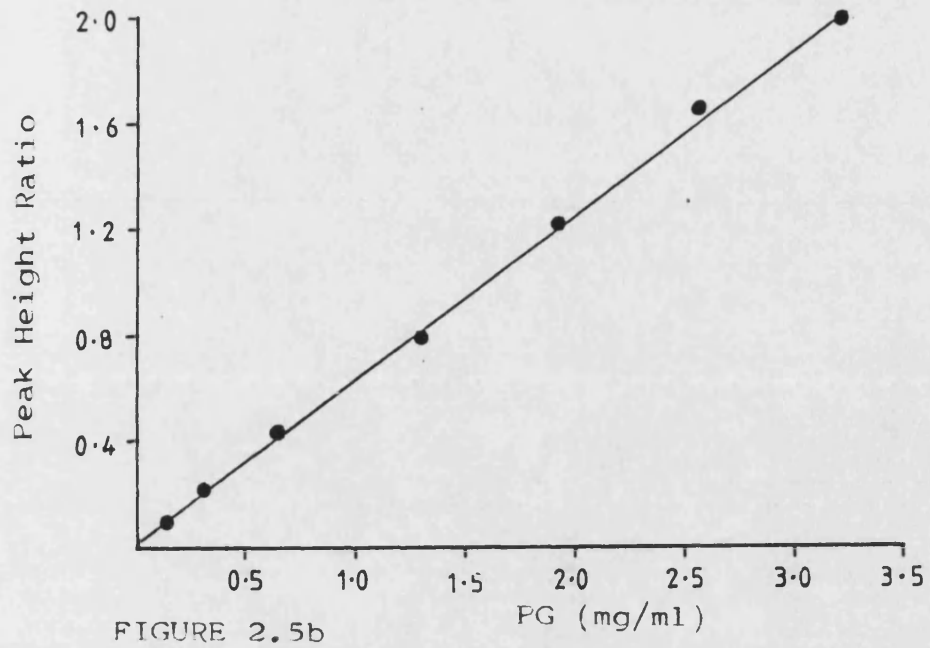
17β-estradiol was assayed in human plasma using an estradiol direct Radioimmunoassay (RIA) kit (purchased from Sorin Biomedica).

Assay Procedure

50 µl of plasma sample was added to 100 µl of tracer (^{125}I-17β -estradiol) and 100 µl of antiserum (17β -estradiol antibody) and

FIGURE 2.5a

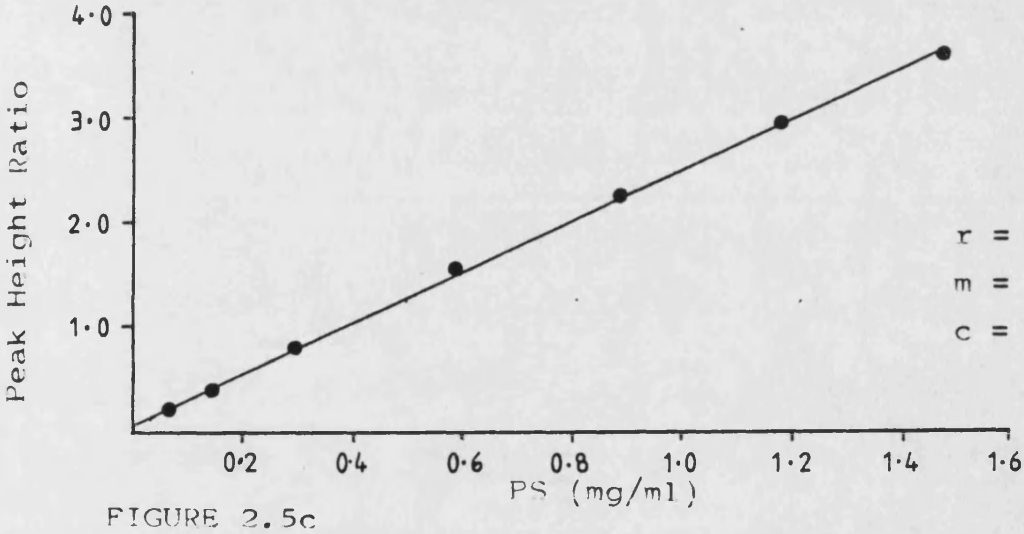
Calibration curve for paracetamol glucuronide (PG) in human urine.



r = 0.9992
m = 0.624
c = 0.013

FIGURE 2.5b

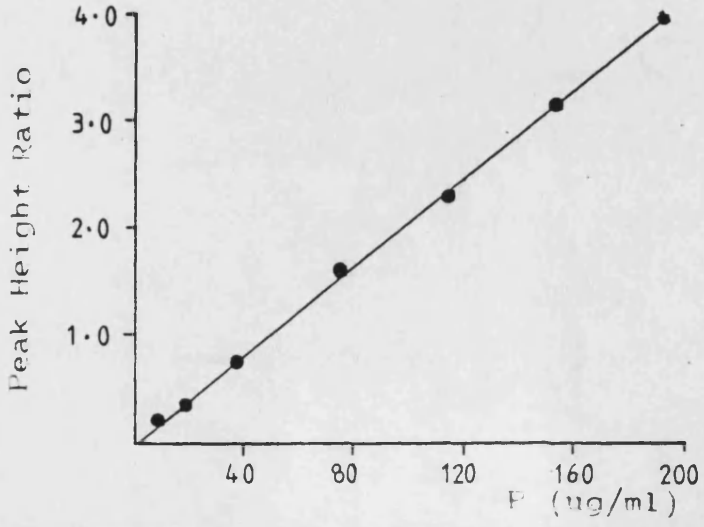
Calibration curve for paracetamol sulphate (PS) in human urine.



r = 0.9986
m = 2.436
c = 0.053

FIGURE 2.5c

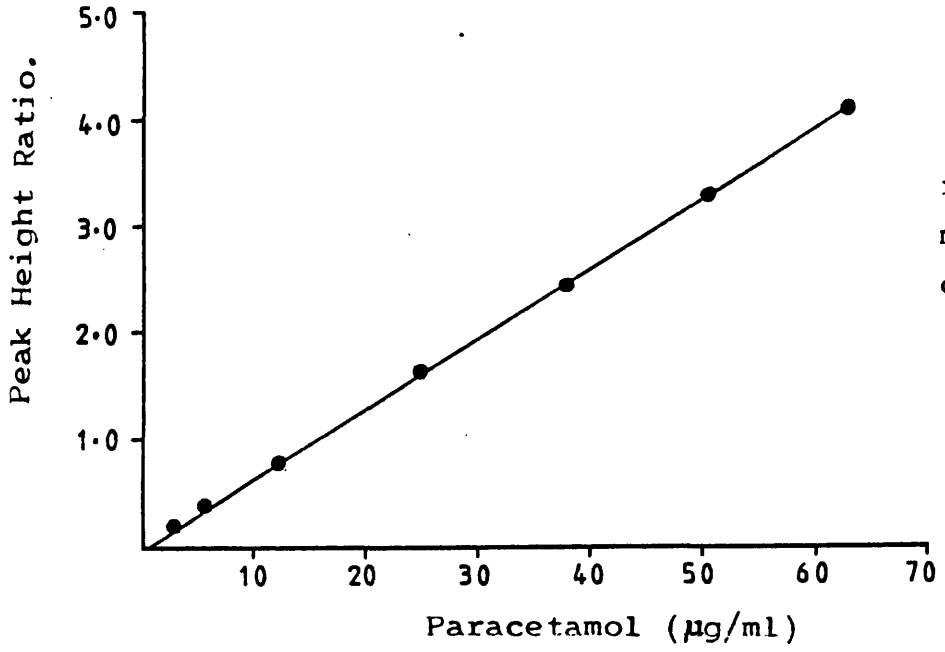
Calibration curve for paracetamol (P) in human urine.



r = 0.9998
m = 0.021
c = 0.013

FIGURE 2.6a

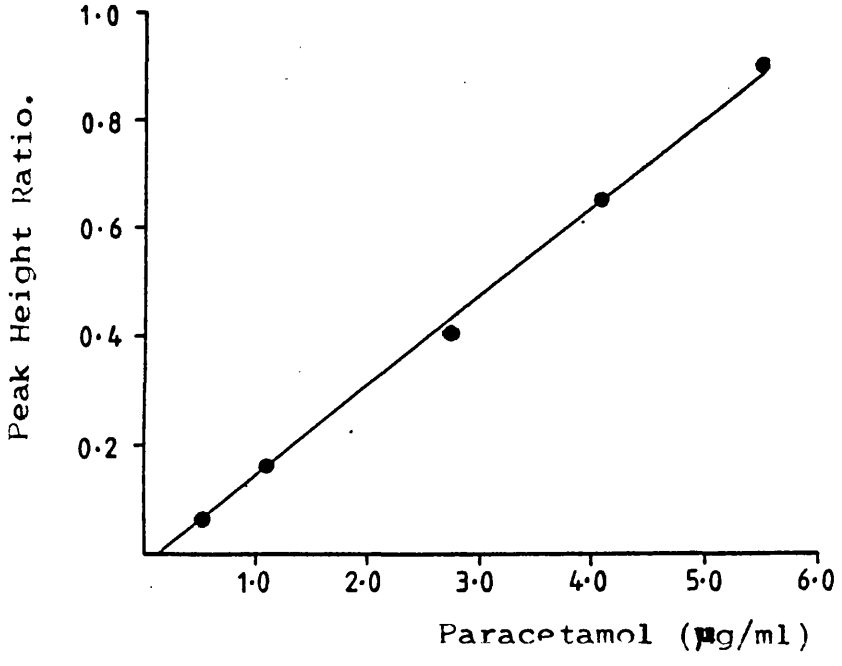
Calibration curve for paracetamol in rat serum.
(High Concentration)



r = 0.9995
m = 0.065
c =-0.023

FIGURE 2.6b

Calibration curve for paracetamol in rat serum.
(Low concentration)



r = 0.9973
m = 0.165
c =-0.021

the contents mixed with a vortex mixer. The tubes were incubated overnight at room temperature. The following morning, 1 ml of precipitating reagent obtained from the kit was added to each tube which was then mixed with a vortex and left to stand at room temperature for 15 minutes. The tubes were centrifuged at 4000 rpm for 20 minutes and the supernatant discarded by inversion. The radioactivity of the precipitate was finally measured using the gamma counter.

Kit Specificity

The antiserum used in the text shows cross-reactivity as follows:

Estradiol	100%
Estrone	0.7%
Estriol	0.55%
20-OH Progesterone	7×10^{-3}%
Ethinyl estradiol	8×10^{-3}%
Cortisol	2×10^{-3}%
Cortisone	
Cortexdone	
Corticosterone	
17-OH Progesterone	
Etiocholanolone	
Pregnenolone	
Androstenedione	
Testosterone	
Dehydroepiandrosterone	
Deoxycortisone	

b) In Urine

17β-estradiol was analysed in human urine using the estradiol radioimmunoassay kit as for the plasma samples.

Assay Procedure

Prior to use of the kit, estradiol glucuronide and estradiol sulphate were enzymatically deconjugated. The concentration of these metabolites was determined by assaying total estradiol concentration before and after deconjugation.

A 5 ml aliquot of urine was taken and its pH altered to pH 5.0 using 1.0 N hydrochloric acid. To 2 x 0.75 ml aliquots, 0.75 ml glucurase was added. To a further 2 x 0.75 ml aliquots 10 mg sulfatase was added and as this enzyme has glucuronidase activity 0.75 ml of 0.02 M saccharate lactone (a glucuronidase inhibitor) was also added. The glucurase-treated, sulfatase-treated, and blank urine were left to incubate at 37° for 24 hours.

Following incubation each aliquot had its pH re-altered to pH 7.4 (± 0.05) using 1 N sodium hydroxide and was then assayed for estradiol. The assay procedure for estradiol in urine was identical to that given in 2.9(a) with a 50 µl urine sample replacing the 50 µl serum sample.

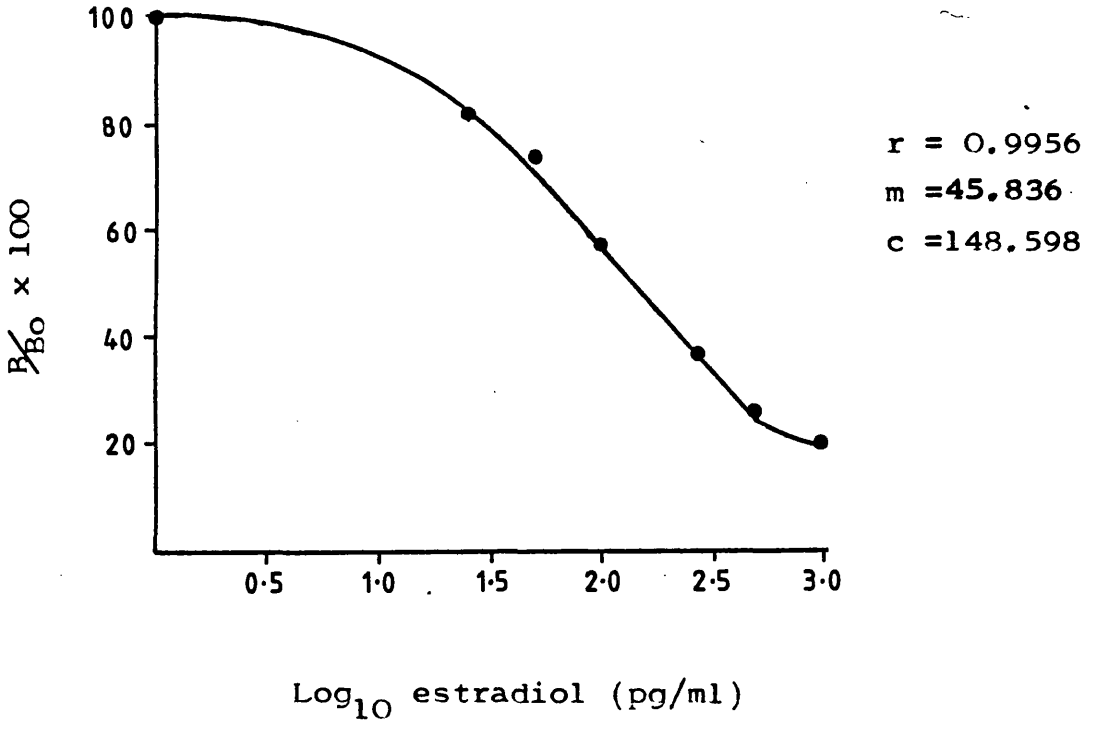
The calibration curve for estradiol was linear over the range 25 to 500 pg/ml as shown in Fig. 2.7.

2.10 DETERMINATION OF RADIOACTIVITY IN BIOLOGICAL SAMPLES

Total radioactivity of urine and serum samples; kidney, liver,

FIGURE 2.7

Calibration curve for estradiol



small and large intestinal and faecal homogenates were measured using the LKB Wallac 1215 Liquid Scintillation Counter. All of these measurements were performed on the day the animal was sacrificed. The kidney, liver, small and large intestinal and faecal homogenates were prepared by homogenising the total tissue in N-saline (0.9% NaCl) using a Thyristor Regler TR50.
The following respective volumes of N-saline were added to the tissues prior to homogenisation: 10 ml; 20 ml; 20 ml; 30 ml; 10 - 30 ml.
100 µl of the homogenate or urine/serum sample was added to 10 ml liquid scintillant, Optiphase. The vials were capped, mixed and counted for 120 seconds. Each sample was measured in duplicate.

2.11 QUANTITATION OF ASPIRIN METABOLITES, PARACETAMOL AND ITS METABOLITES AND ESTRADIOL IN BIOLOGICAL FLUIDS USING STANDARD CURVES

a) Preparation of Standard Curves

i) Aspirin and paracetamol metabolites

Five to eight urine or serum samples spiked with a range of solute concentrations plus a constant concentration of the respective internal standards were assayed in duplicate by HPLC. The peak heights for aspirin metabolites in urine and paracetamol and its metabolites in both urine and serum were determined together with the peak heights of the internal standards, and the peak height ratio calculated (i.e. peak height of the metabolite/peak height of the internal standard). Standard curves were checked throughout and

no significant variation was observed.

The data was fitted to the following equation using the linear least-squares regression method:

$$y = mx + c$$

where y = peak height ratio

x = concentration of metabolite

m and c = regression coefficients

ii) Estradiol

Seven urine or serum samples spiked with a range of estradiol concentrations were assayed in duplicate by radioimmunoassay. The net counts (total CPM - background CPM) were determined for each of the standards and expressed as a percentage of the zero estradiol standard:

$$\frac{\text{net count for standard (B)}}{\text{mean net count for zero standard } (B_o)} \times 100 = \frac{B}{B_o}\ \%$$

Data was fitted to the following equation - the linear least-squares method:

$$y = mx + c$$

where $y = B/B_o\%$

$x = \text{Log}_{10}$ (concentration of estradiol)

m and c = regression coefficients

b) Linearity of Calibration Curves

From the calibration curve for each metabolite, the correlation coefficient r, the gradient, m and the intercept, c were calculated

using the linear least-squares regression method. For each calibration curve, every concentration point was obtained from an independent dilution.

c) Concentration Determination

Peak height ratios of the parent drug and/or its metabolites for aspirin and paracetamol were determined in serum and urine samples and concentrations were calculated using the equation:

$$x = \frac{y - c}{m}$$

where x = unknown concentration to be determined

y = peak height ratio

m and c = regression coefficients calculated from the calibration curves

The net count for total estradiol in urine and serum samples was determined and concentrations were calculated using the equation:

$$\text{Log}_{10}x = \frac{y - c}{m}$$

where x = unknown concentration to be determined

y = $B/B_o\%$

m and c = regression coefficients calculated from the calibration curves

All urine samples were diluted to ensure that they fell in the linear portion of the calibration curve.

2.12 STATISTICAL ANALYSIS AND TREATMENT OF DATA

Results were expressed as the mean ± standard error of the mean. Further statistical analysis was performed using the computer statistical package, Minitab. Statistical difference was determined using either the student's t test (two-tailed, unpaired) or the Mann-Whitney test with statistical significance occurring at a probability of $p < 0.05$. For the t-test to be used 2 basic assumptions have to be fulfilled:

1) The distribution is not too markedly skewed, and
2) Population variances are equal, or
3) If population variances are unequal, then $n_1 + n_2 > 60$ and $n_1 \equiv n_2$.

If these requirements were not satisfied then the Mann-Whitney test was used.

Probit plots were performed on all population distribution distribution data (Finney, 1971). A non-linear probit plot assumes deviation from a normal population. Deviation from linearity was determined by the x^2-test, with statistical significance occurring at a probability of 0.05. A statistical significant x^2 indicates a non-linear probit plot which in turn indicates deviation from a normal distribution.

The absolute quantitites of the paracetamol and aspirin metabolites in urine were expressed as the equivalent weight of paracetamol and salicylic acid respectively using the following conversion factors:

SUA (mg) x 0.708	SUA as the equivalent weight of SA
PS(mg) x 0.657	PS as the equivalent weight of P
PG(mg) x 0.462	PG as the equivalent weight of P

(This treatment was not necessary for the estradiol data as absolute quantities of estradiol metabolites were not determined).

CHAPTER THREE

ASPIRIN METABOLISM IN HUMAN VOLUNTEERS

3.1 INTRODUCTION

Large interindividual differences in the serum concentration of salicylic acid (SA) and in salicylate excretion rates have been widely reported (including Caldwell *et al.*, 1980; Levy and Hollister, 1964; Paulus *et al.*, 1971; Gupta *et al.*, 1975). Several studies have been performed in order to determine the cause(s) of this variation. Sex differences in salicylate metabolism have been found, however there is conflicting evidence concerning the effect of age on, and genetic control of, salicylate metabolism (Ho *et al.*, 1985; Oldham, 1983; Evans and Clarke, 1961; Furst *et al.*, 1977). By giving a therapeutic dose of aspirin to a large number of healthy adult volunteers and studying the excretion of salicylic acid and its major metabolite, salicyluric acid (SUA), the effect of **both** genetic **and** environmental factors on the glycine conjugation of salicylate was examined.

3.2 PROCEDURE

150 volunteers; 88 male, 62 female, were given aspirin (600 mg), which was taken in the morning after first voiding the bladder. Urine was collected for 0 - 8 hours and the urinary metabolites salicylic acid (SA) and salicyluric acid (SUA) were assayed by HPLC as described in 2.7. The quantity of SUA excreted is expressed as

the equivalent weight of SA, using the correction factor given in 2.12.

The age range for males was 18 to 61 years with a mean (± SE) age of 33.3 ± 1.5 years; the weight range was 60 to 102 kg with a mean weight of 72.9 ± 1.0 kg. The age range for females was 18 to 59 years with a mean age of 26.9 ± 1.4 years; the weight range was 44 to 90 kg with a mean weight of 60.4 ± 1.2 kg.
The student's t test was used as the test for significance throughout this study.

3.3 RESULTS

a) Environmental control of aspirin metabolism

Effect of urine pH

Urine pH has been well documented to affect salicylic acid excretion (Macpherson *et al.*, 1955; Gutman *et al.*, 1955) and such an effect was found in this study. When total salicylate excreted in 8 hours was plotted against urine pH for the 150 subjects (Fig. 3.1) a highly significant positive correlation was found ($r = 0.732$; $p < 0.001$). The best fitting curve describing this relationship between urine pH and SA excretion was determined by computer analysis.

$$y = a^* x^b$$

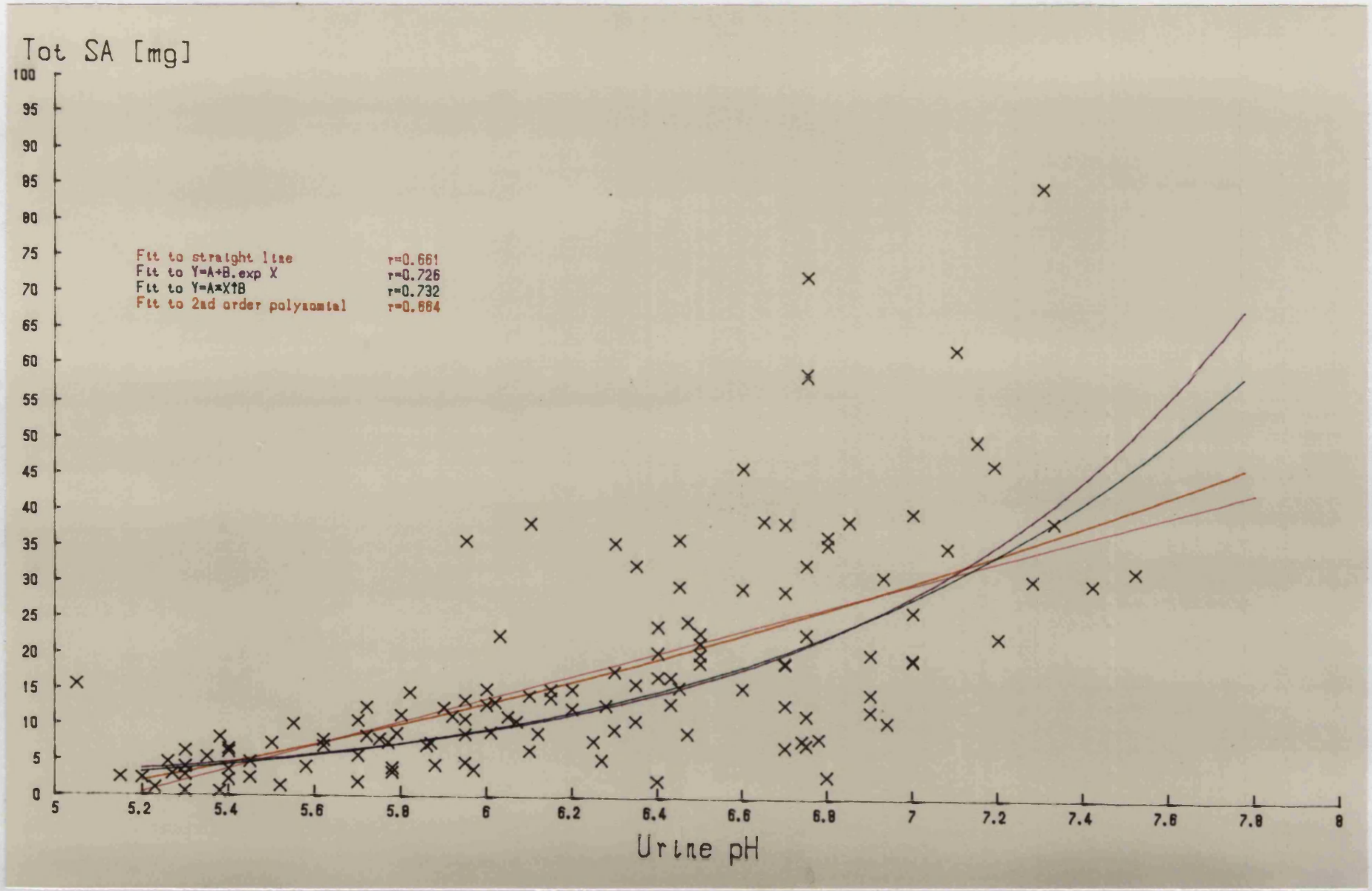
where x = urine pH

y = total SA excreted in 8 hr.

a,b = regression coefficients

FIGURE 3.1

Effect of urine pH on the 0.8 hr urinary excretion of SA.



In order to determine the influence of other environmental and genetic factors on salicylate metabolism, the effect of pH had to be removed. This was done by correcting the total salicylate excretion at the pH observed, to a standardised pH of 6.2, the mean pH, using the method given below.

Correction Method

As the urine pH for each subject was known, the expected SA excretion for that pH was calculated using the formula:

$$y = a^* x^b$$

where y = salicylate excreted to be determined

x = urine pH

$a = 2.613 \times 10^{-5}$

b = 7.14

The salicylate excretion for the standardised, mean pH of 6.2 was also calculated:

$$\text{SA excretion} = 2.613 \times 10^{-5} * (\text{pH } 6.2^{\frac{7.14}{}})$$

$$= 11.9$$

The corrected value for total salicylate excretion was then determined as follows:

$$\underset{\text{excretion}}{\text{Corrected SA}} = \underset{\text{excretion}}{(\text{Observed SA}} - \underset{\text{excretion}}{\text{Expected SA)}} + \underset{\text{at PH 6.2}}{\text{SA excretion}}$$

In this way the majority of the variation in SA excretion due to urine pH was removed. Therefore, any variation remaining is probably due to factors other than urine pH.

ALL VALUES FOR SA EXCRETION ARE EXPRESSED AS CORRECTED SA VALUES UNLESS OTHERWISE STATED

Effect of age and weight

Table 3.1 gives the total amount and percentage dose of corrected SA and SUA excreted in 8 hours following a therapeutic dose of 600 mg ASA. 15.01 ± 0.91 mg (3.26 ± 0.20%) of SA were excreted together with 193.4 ± 5.0 mg (42.24 ± 1.09%) of SUA. Individual results (expressed in mg) are given in Appendix 1 together with further details of the volunteers including sex, age, weight, smoking habits and drugs taken.

Table 3.1. Urinary recovery (0 - 8 hr) of SA and SUA following therapeutic dose in 150 normal volunteers.

Aspirin metabolite	Total excreted Mean ± SE	% of dose Mean ±SE	Range
SA	15.01 ± 0.91 mg	3.26 ±0.20%	0 - 59.9 mg
SUA	193.4 ± 5.0 mg	42.04 ± 1.09%	28.2-444.4 mg

No correlation was found between age and excretion of the 2 aspirin metabolites, SA and SUA as shown in Figs. 3.2a and b ($r = 0.005$, 0.088 respectively, $p > 0.1$). Similarly, body weight and excretion of these 2 metabolites also failed to show a correlation as can be seen in Figs. 3.3 a and b ($r = -0.204$, 0.342 respectively, $p > 0.1$).

Effect of gender

Urinary excretion of SA and SUA were compared in males and females and significant differences were found. Males excreted 213.6 ± 5.04

FIGURE 3.2a

Effect of age on excretion of SA (pH corrected) in 0-8 hr urine.

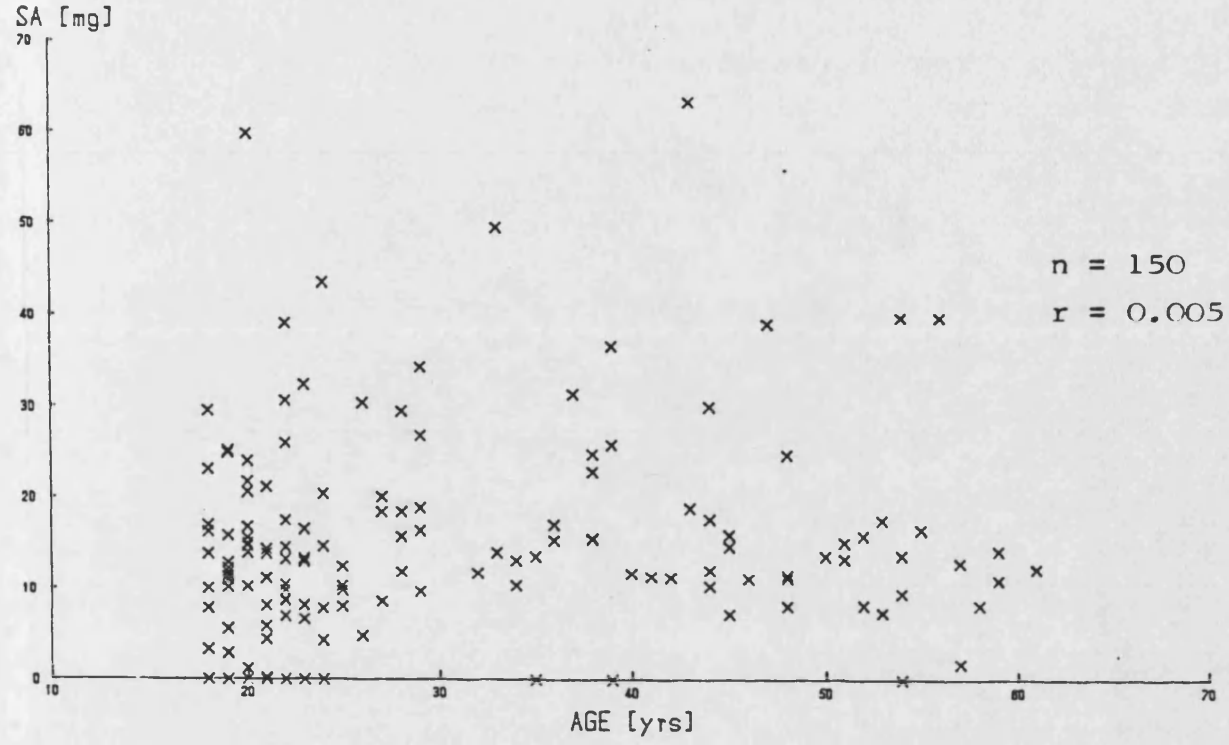


FIGURE 3.2b

Effect of age on excretion of SUA in 0-8 hr urine.

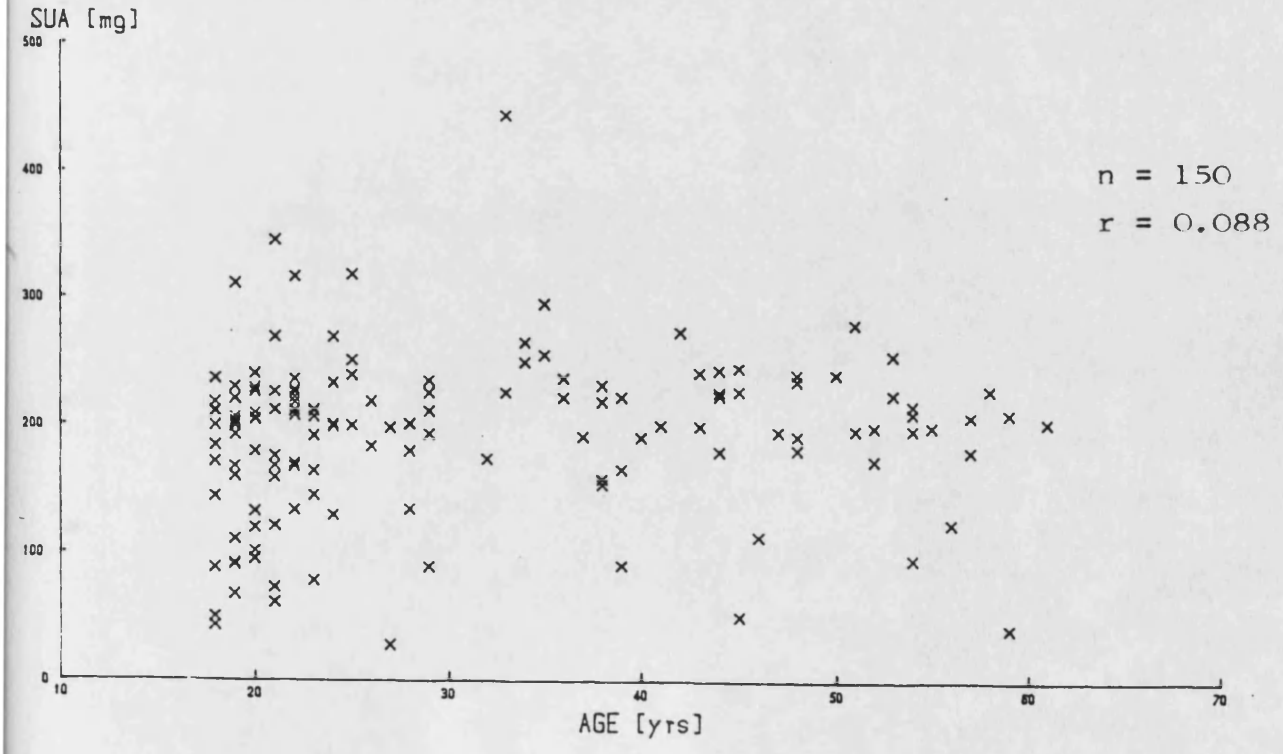


FIGURE 3.3a

Effect of body weight on excretion of SA (pH corrected in 0-8 hr urine

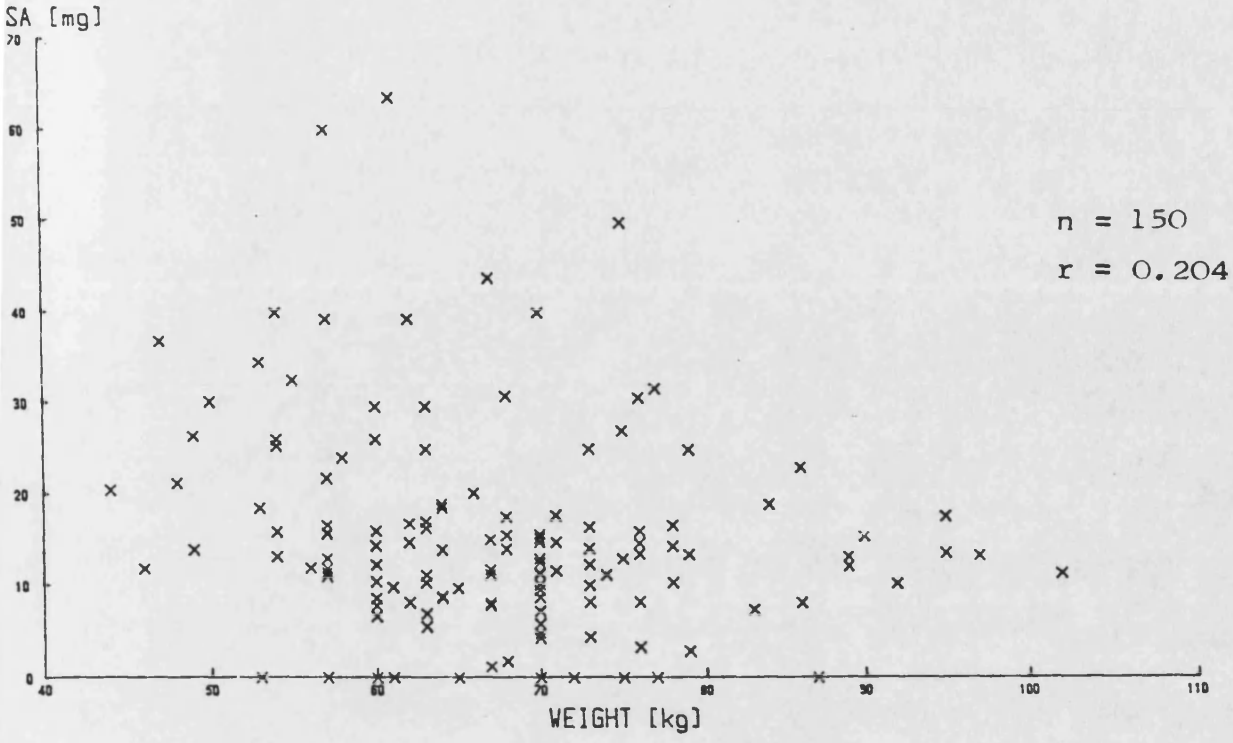
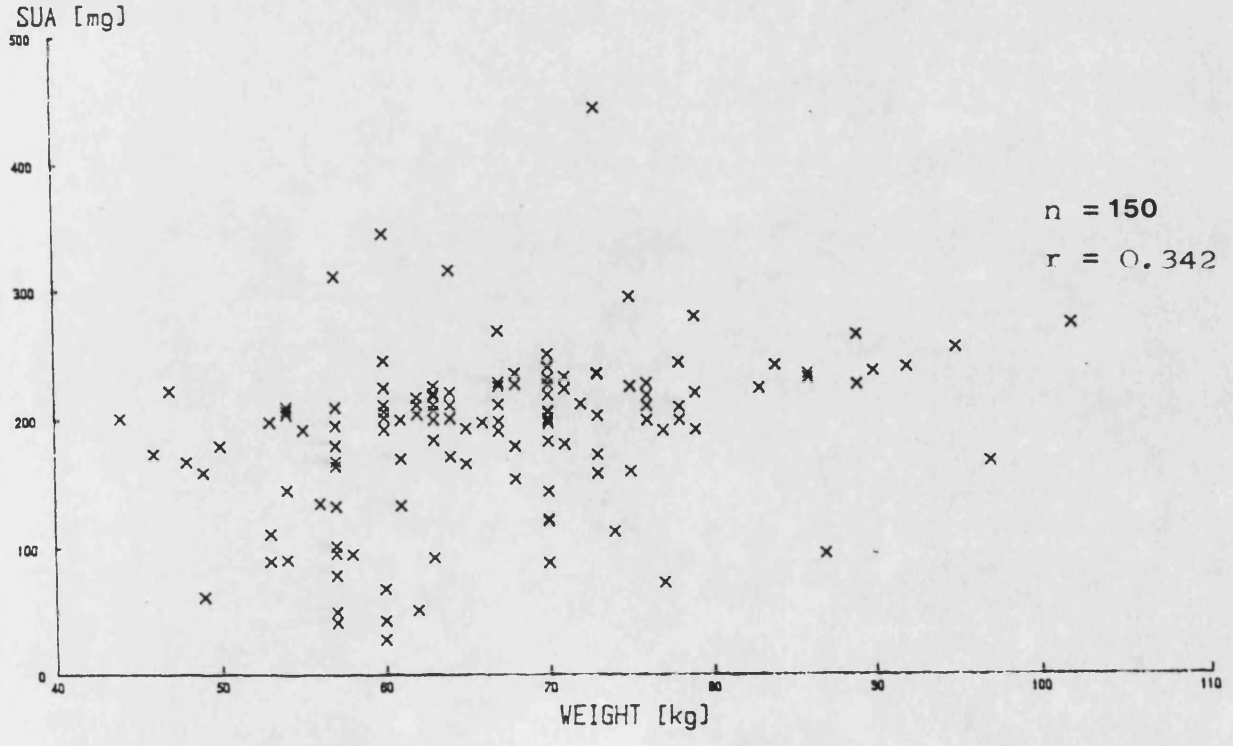


FIGURE 3.3b

Effect of body weight on excretion of SUA in 0-8 hr urine.



of the major metabolite SUA whilst females excreted a significantly lower amount of 163.1 ± 8.65 mg of SUA ($p < 0.01$).
For the minor metabolite, SA, males excreted 13.1 ± 1.0 mg whilst females excreted a significantly higher amount of 17.8 ± 1.65 mg ($p < 0.01$). Figs 3.4a and b show the 8-hour urinary excretion of SA and SUA in both males and females.

Effect of smoking and oral contraceptive steroids

The excretion of the 2 ASA metabolites, SA and SUA was compared in smokers and non-smokers for both male and female. No significant difference was found between smokers and non-smokers in any of the groups studied, as shown in Figs. 3.5a and b and Table 3.2.

Table 3.2. Excretion of SA and SUA (mean ± SE) in smokers and non-smokers.

Aspirin metabolite	Male (mg)		Female (mg)	
	Smoker (n=23)	Non-smoker (n=56)	Smoker (n=7)	Non-smoker (n=48)
SA	12.6±2.4	13.1± 1.2	22.1± 5.8	17.5±1.95
SUA	207.9±9.55	216.8±6.7	135.45±30.3	166.3±9.9

For females using oral contraceptive steroids, OCS, (n = 10) and females not using oral contraception (n = 45) the excretion of SA and SUA was compared. OCS users excreted 159.25 ± 7.0 mg SUA and 17.2 ± 2.7 mg SA. Non-users excreted 163.1 ± 10.1 mg SUA and

FIGURE 3.4a

Excretion of salicyluric acid (SUA) in 0-8 hr urine in males and females

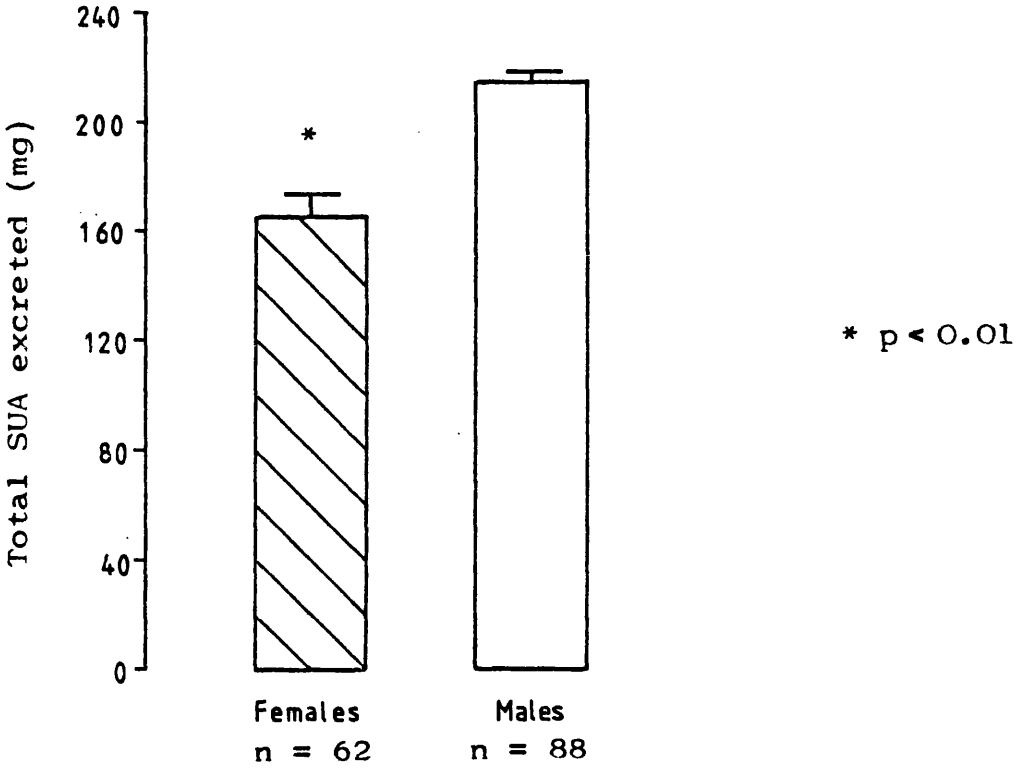


FIGURE 3.4b

Excretion of salicylic acid (SA) in 0-8 hr urine in males and females

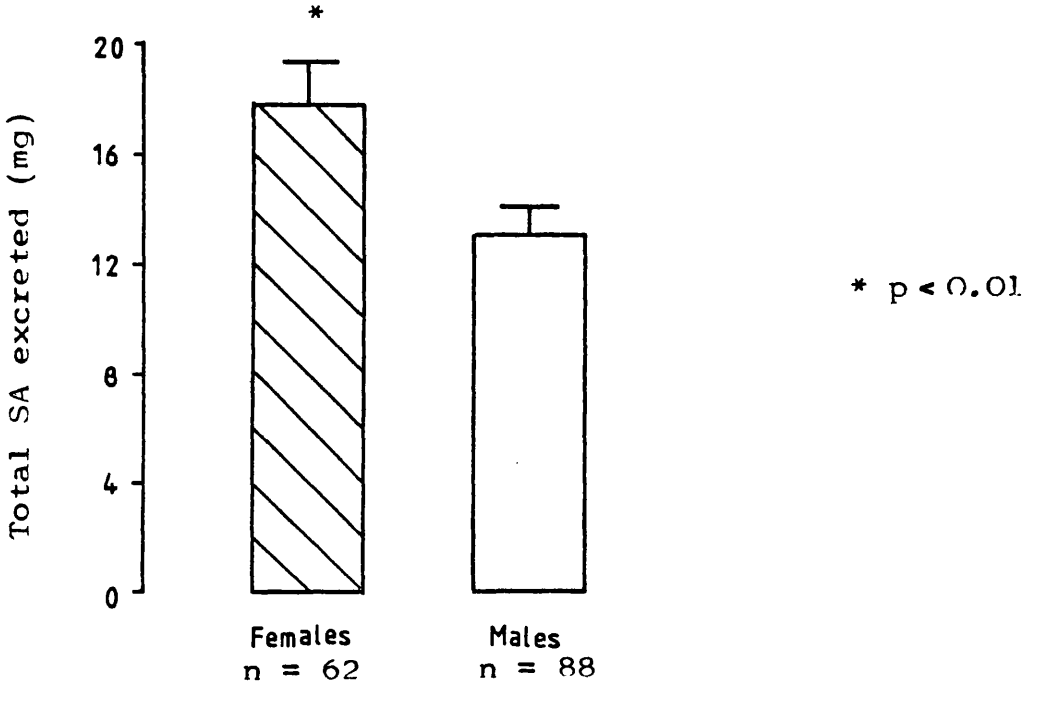


FIGURE 3.5a

Effect of smoking on the excretion of salicyluric acid (SUA) in the 0-8 hr urine.

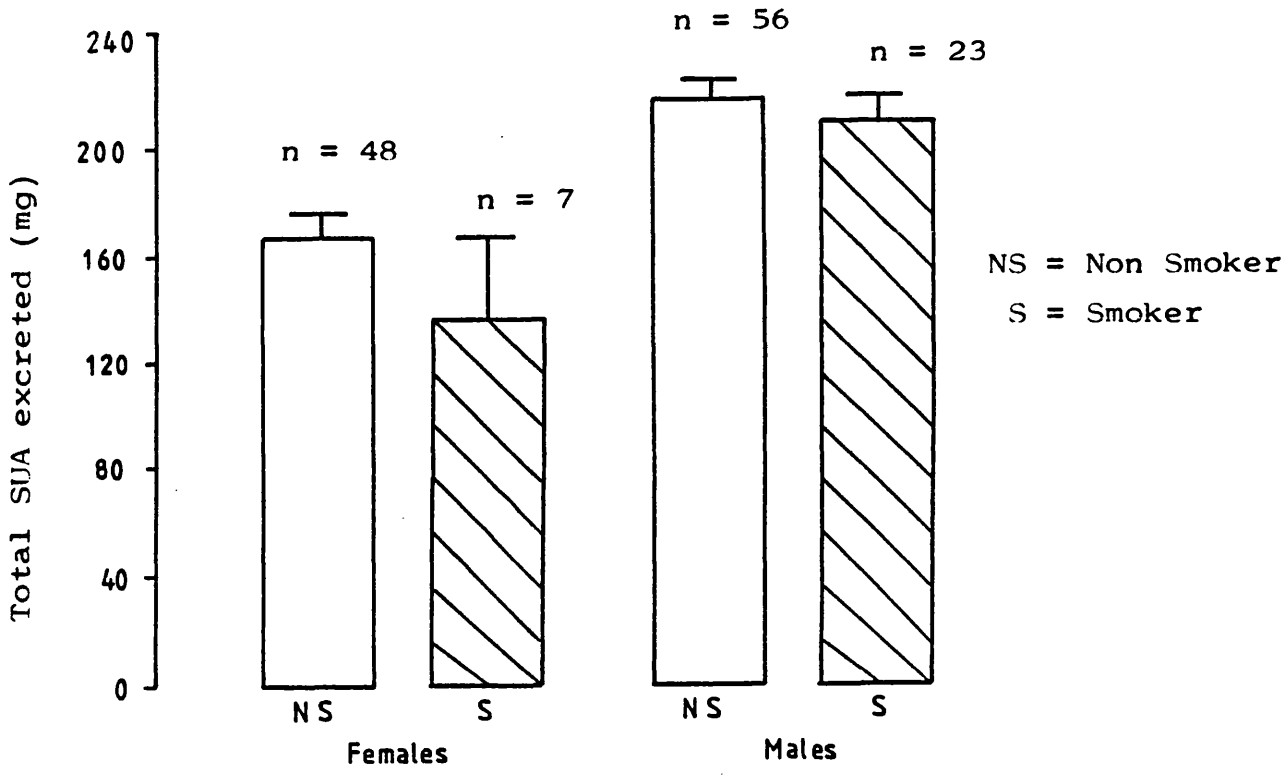


FIGURE 3.5b

Effect of smoking on the excretion of salicylic acid (SA) in the 0-8 hr urine.

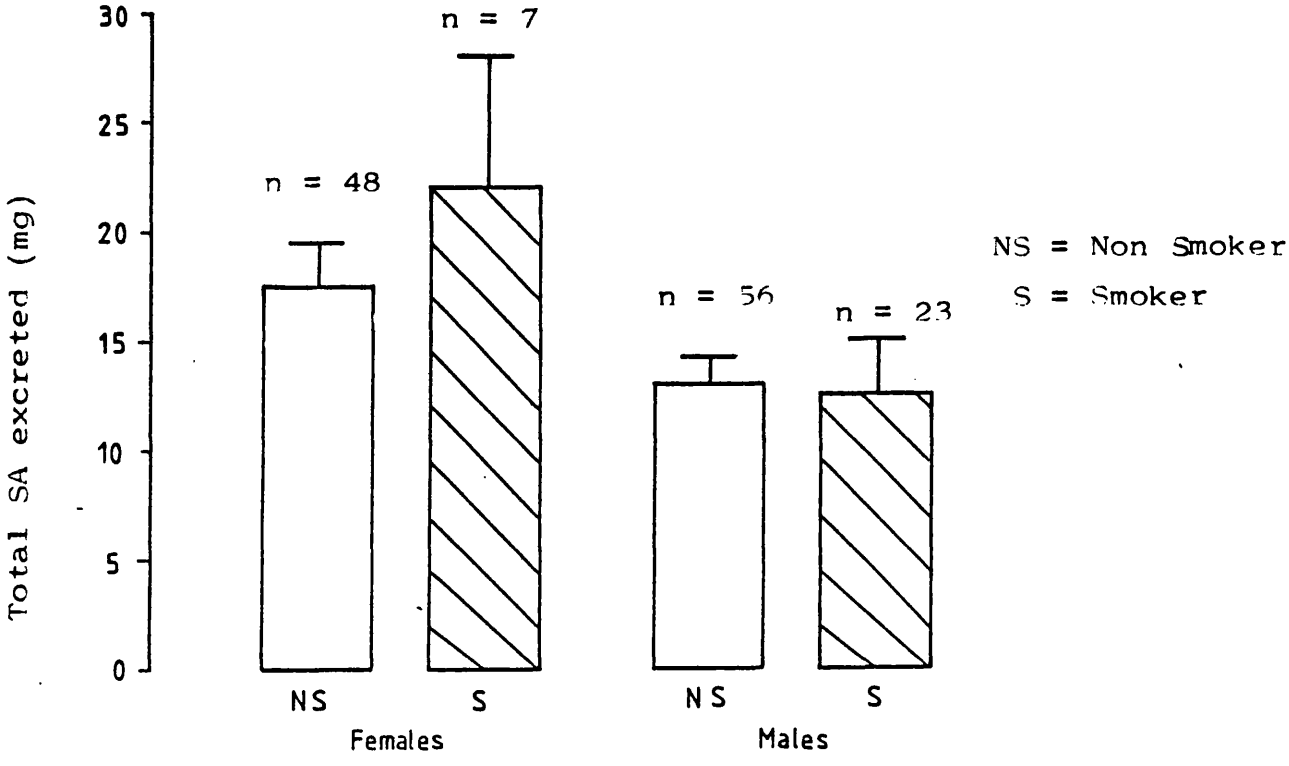


FIGURE 3.6a

Effect of OCS on salicyluric acid (SUA) excretion in 0-8 hr urine

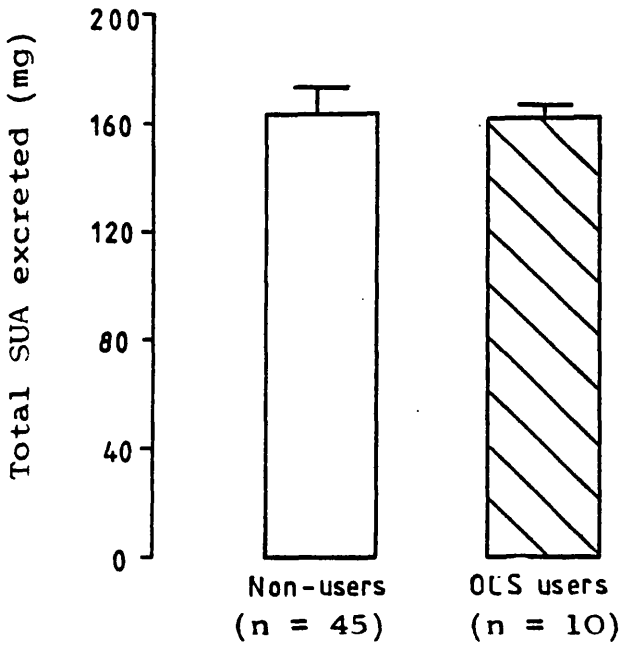
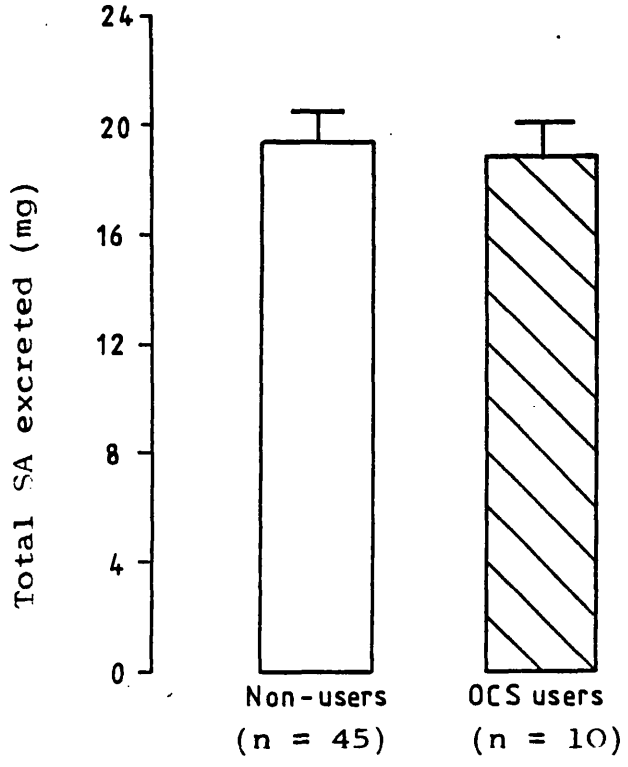


FIGURE 3.6b

Effect of OCS on salicylic acid (SA) excretion in 0-8 hr urine.



18.3 ± 2.2 mg SA. These differences were found to be non-significant as shown in Figs. 3.6a and b.

b) Genetic control of aspirin metabolism

Figs. 3.7a and b show the frequency distribution histograms and probit plots for the 8-hour urinary excretion of observed SA and corrected SA respectively. The distribution of observed SA excretion appeared to be strongly positively skewed as seen in the frequency distribution histogram. This skewness is confirmed in the probit plot which was found to be non-linear ($x^2 = 39.7$, $p < 0.01$). The distribution of corrected SA excretion appeared to be approaching a normal distribution when the frequency distribution histogram was studied. However, the probit plot for this data was found to be non-linear ($x^2 = 39.1$, $p < 0.01$) indicating that the distribution may be skewed.

Fig. 3.8 shows the frequency distribution histogram and probit plot for the 0 to 8-hour urinary excretion of SUA. The frequency distribution histogram showed a negative skewness for the distribution of SUA excretion. However, the probit plot only just failed the test of linearity ($x^2 = 29.4$, $p < 0.05$) and so the distribution is probably approaching normality and is only slightly skewed.

The Metabolic Ratio (MR) was calculated for each individual as follows:

$$\text{Metabolic Ratio} = \frac{\text{Excretion of pH corrected SA (mg)}}{\text{Excretion of SUA (mg)}}$$

Fig. 3.9 shows the frequency distribution histogram and probit plot for the metabolic ratio of salicylic acid. The histogram shows a

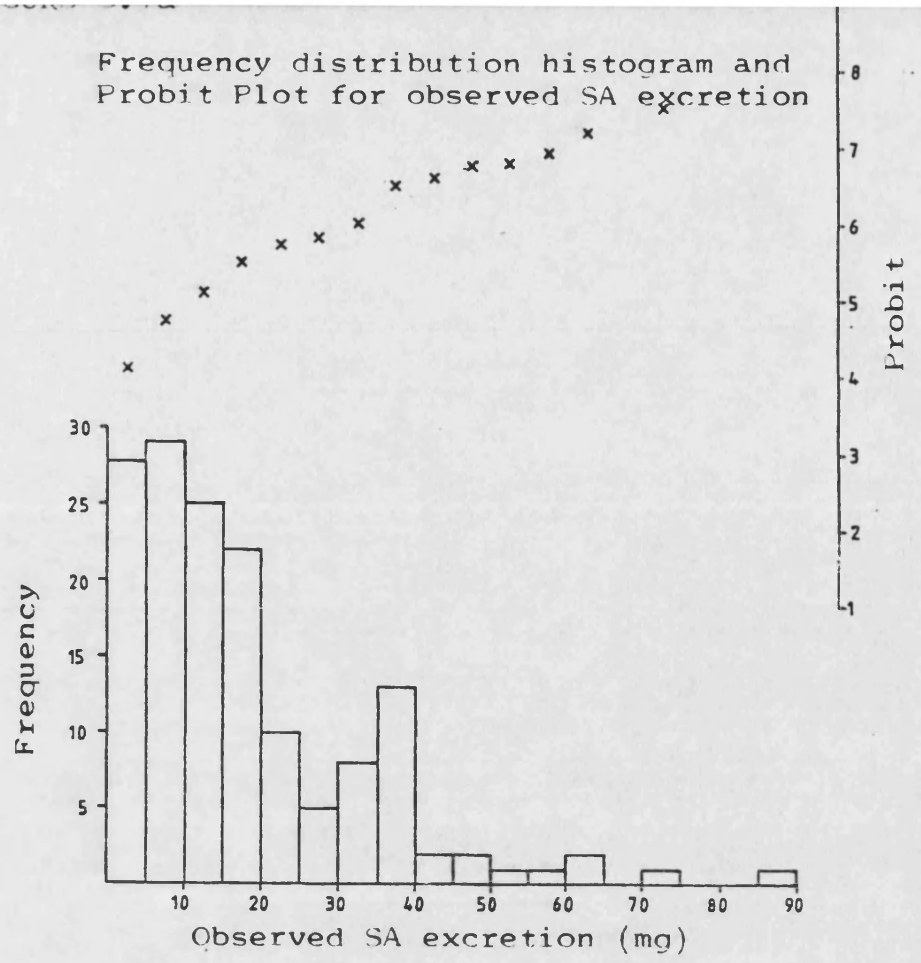

Frequency distribution histogram and
Probit Plot for observed SA excretion
Frequency
Probit
Observed SA excretion (mg)

FIGURE 3.7b

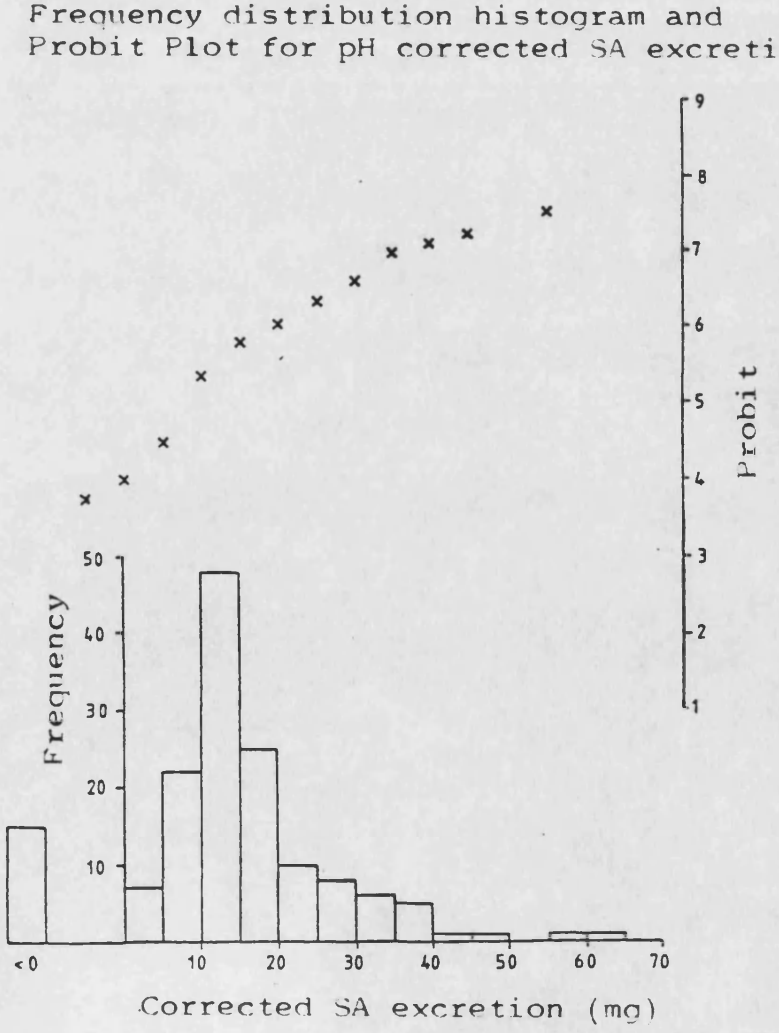

Frequency distribution histogram and
Probit Plot for pH corrected SA excretion
Frequency
Probit
Corrected SA excretion (mg)

FIGURE 3.8

Frequency distribution histogram and
Probit Plot for SUA excretion

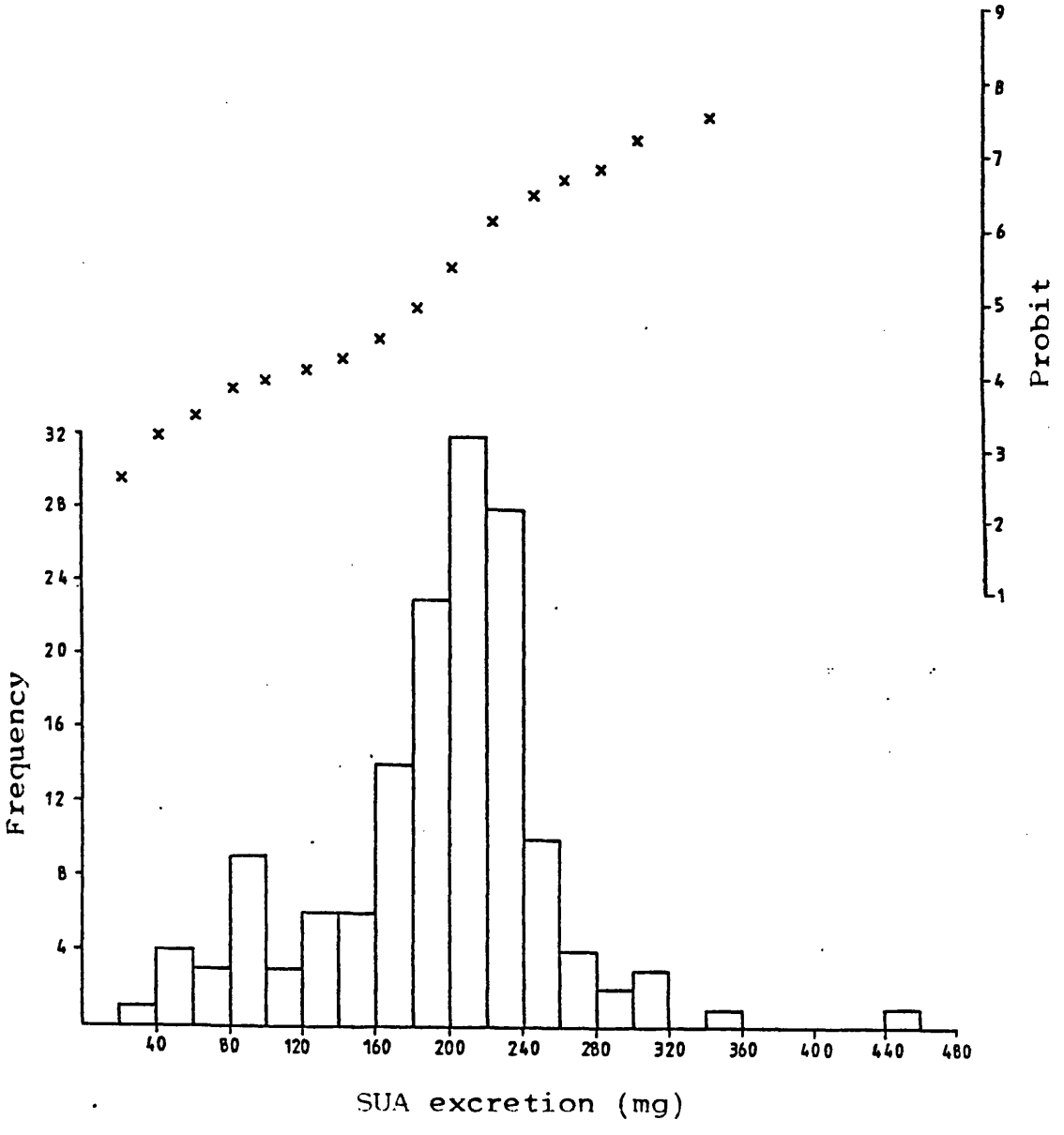
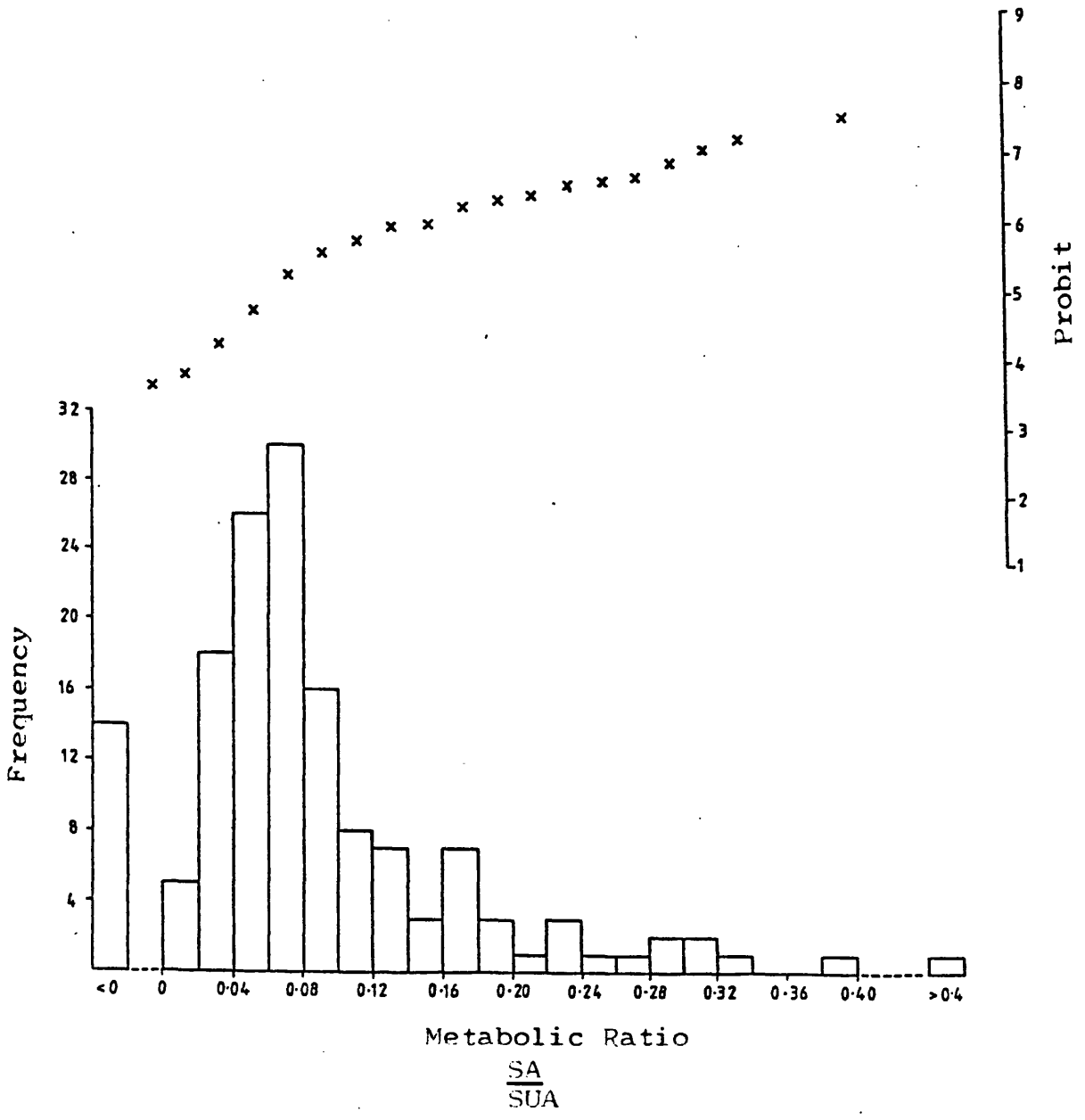


FIGURE 3.9

Frequency distribution histogram and Probit Plot for the metabolic ratio, $\frac{SA \text{ (pH corrected)}}{SUA}$



strongly positively skewed distribution. This skewness is supported by the probit plot which was found to be non-linear (x^2 = 44.1, $p < 0.01$).

Repeat studies

600 mg aspirin was taken a second time by 13 of the volunteers with 6 months to 2 years between the 2 tests. Table 3.3 gives the mean values for excretion of SA, SUA and SA + SUA in the first and second determination. Between the 2 tests, SA showed a 72.6 ± 32.0% change; SUA a 22.8 ± 6.3% change and SA + SUA a 22.1 ± 5.6% change (mean ± SE).

Table 3.3. Urinary recovery (0 - 8 hr) of SA and SUA in 2 separate tests for 13 subjects (mean ± SE)

Aspirin Metabolite	1st determination		2nd determination	
	Total excreted (mg)	% of dose	Total excreted (mg)	% of dose
SA	15.8±4.45	3.4±1.0	20.8±5.4	4.5±1.2
SUA	194.0±11.7	42.2±2.5	188.8±14.6	41.0±3.2
SA + SUA	209.8±9.0	45.6±1.9	209.6±15.7	45.6±3.4

The 1st estimates of the aspirin metabolites, SA (corrected) and SUA are plotted against the 2nd estimates in Figs. 3.10a and b respectively. Figs. 3.11a and b show the 1st and 2nd estimates of SA + SUA and the metabolic ratio, SA/SUA respectively. The spread

FIGURE 3.10a

Reproducibility plot for the percentage dose excreted as free SA.

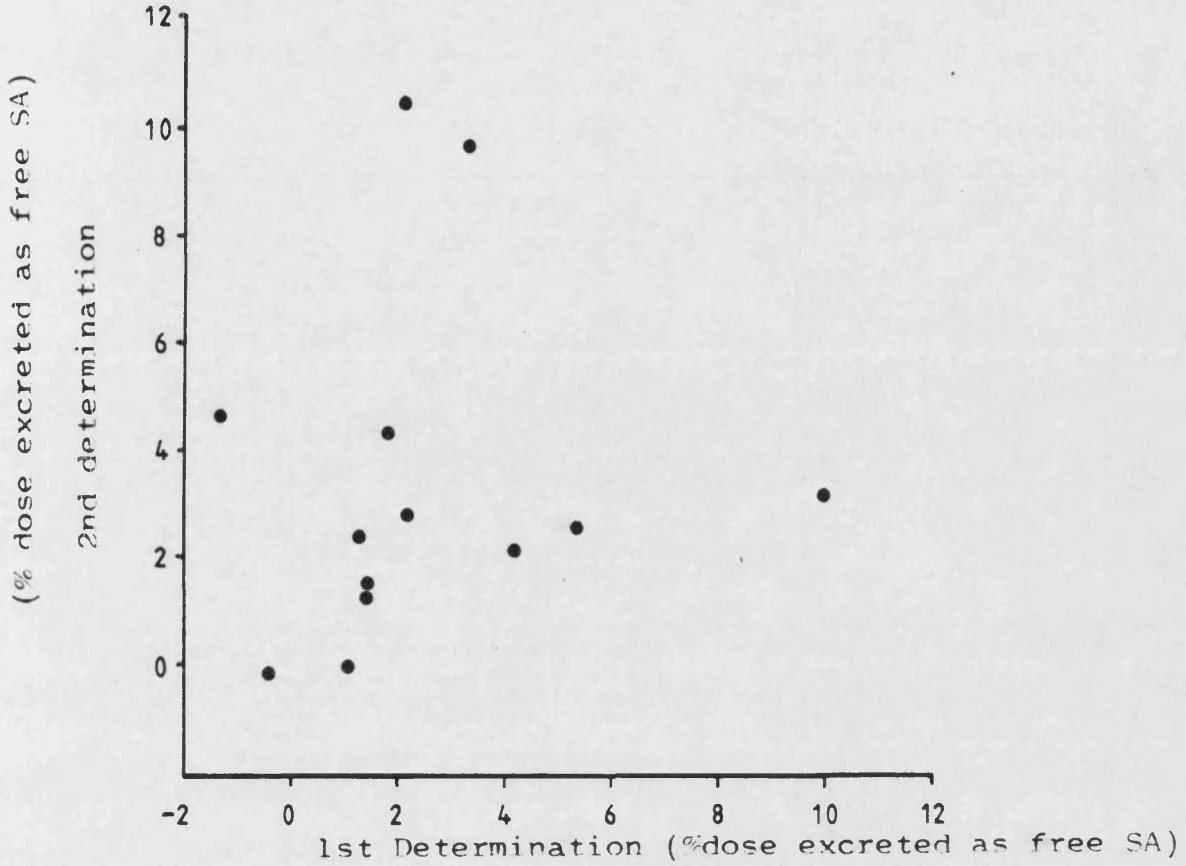


FIGURE 3.10b

Reproducibility plot for the percentage dose excreted as SUA

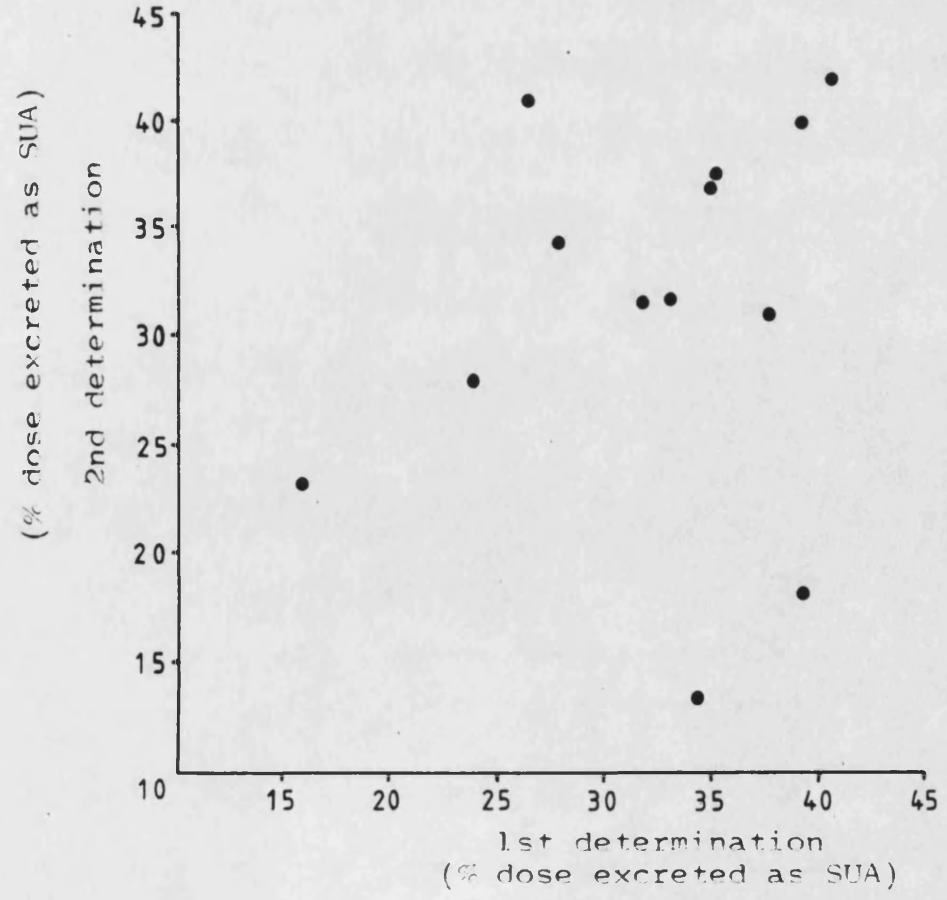


FIGURE 3.11a

Reproducibility plot for the percentage dose excreted as SUA + SA

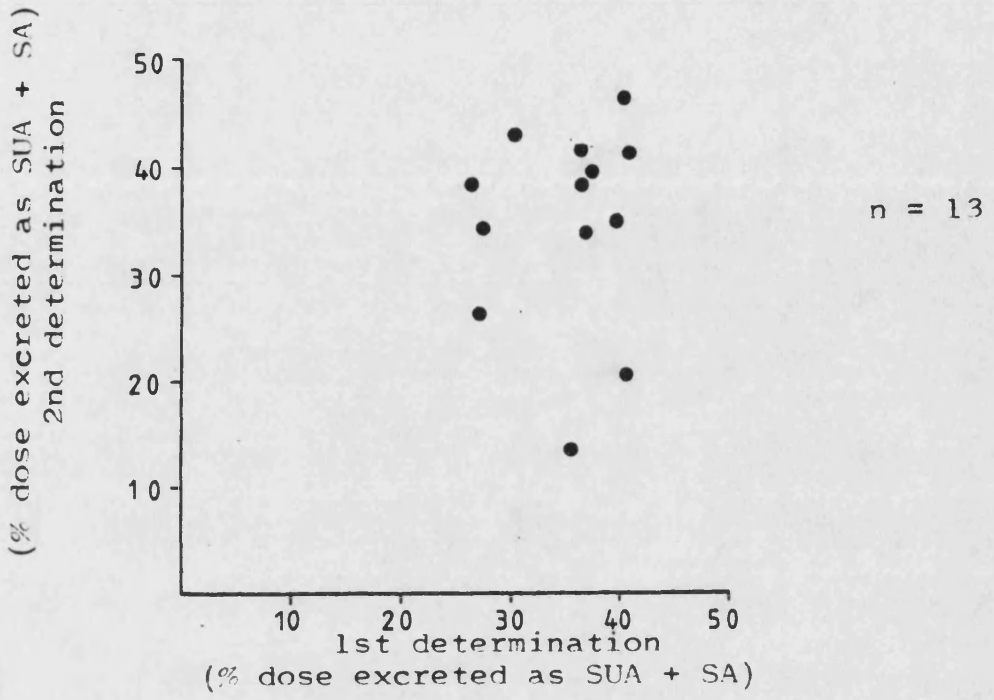
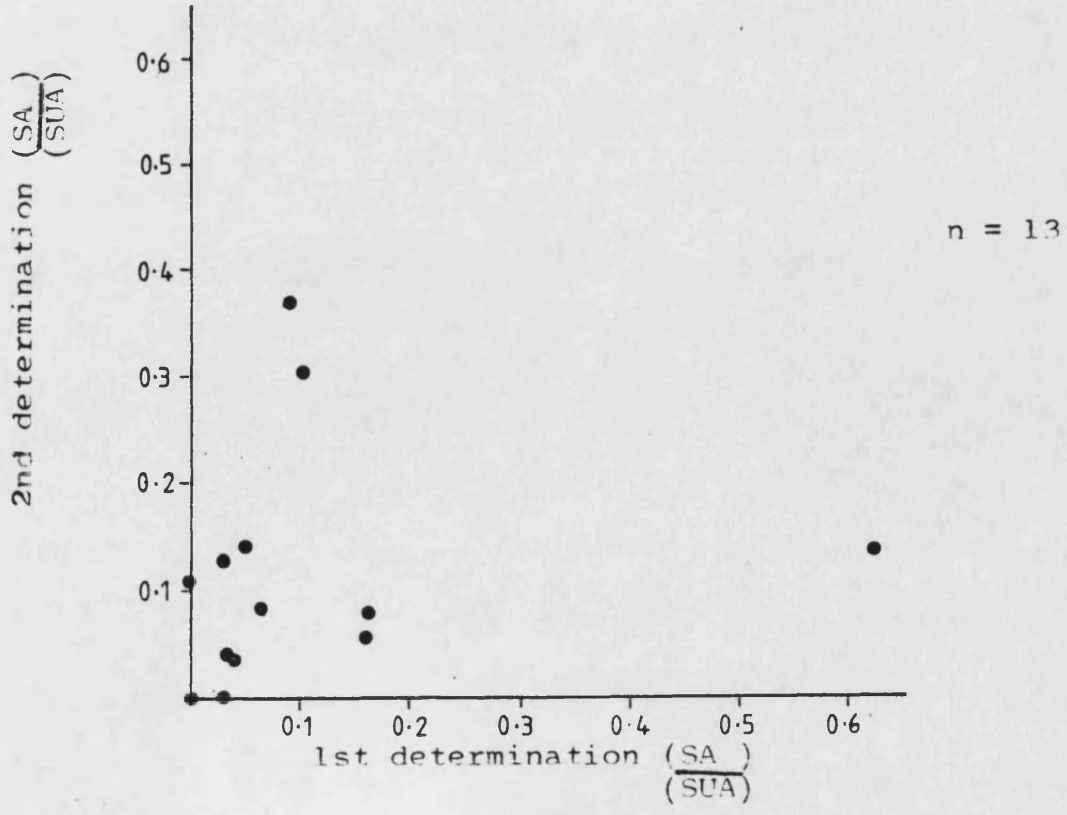


FIGURE 3.11b

Reproducibility plot for the metabolic ratio, $\frac{SA}{SUA}$



of results seen in the reproducibility plots for SA excretion and the metabolic ratio are much greater than the spread seen in the plots for SUA and SA + SUA excretion. SUA and SA + SUA excretion was found to vary by 25% or less in 9 subjects (70% of the volunteers participating in the repeat study) whilst SA excretion and the metabolic ratio, SA/SUA varied by 25% or less in only 2 subjects (15% of the 'repeat' volunteers).

3.4 DISCUSSION

Aspirin metabolism was studied in 150 normal volunteers following a therapeutic (600 mg) dose. After oral administration the 8-hour urine collection was analysed for SA and SUA. A mean of 42 ± 1.1% of the dose was excreted as SUA, with a mean of 3.3 ± 0.2% of the dose being excreted as SA (corrected for pH), which is in agreement with other workers including Bedford *et al.*, (1965), Cummings *et al.* (1966) and Caldwell *et al.* (1979).

Urine pH and salicylic acid excretion

The effect of urine pH on salicylate excretion as found by Macpherson *et al.* (1955) and Gutman *et al.* (1955) who reported a log-linear relationship, was confirmed in the present study. A highly significant positive correlation ($r = 0.732$, $p < 0.01$) between urine pH and observed SA excretion was found and in the pH range studied (pH 5.0 to 8.0) an exponential curve was determined as the best-fit for the data. The difference between this and the earlier reported relationship between urine pH and SA excretion is

probably due to the much larger numbers used in the present study.

A positive correlation between urine pH and SA excretion was expected as the renal clearance of SA is known to involve in addition to glomerular filtration and active secretion, passive back-diffusion into the renal tubules. Only the non-ionised form of SA is subject to tubular reabsorption. Therefore, as urine pH increases, so does the degree of SA ionisation resulting in decreased tubular reabsorption and finally increased urinary excretion of SA. As urine pH increases from 5 to 8, the renal clearance of SA has been found to increase more than 20-fold. As urine pH was known to have such a profound effect on SA excretion, in order to determine if any other factors under study cause any changes in SA excretion, the effect of pH had to be removed. This was done by correcting the salicylic acid excretion for pH whereby all SA values were adjusted to the mean pH of 6.2 using the method given in 3.3a (a method also used by Whittaker and Price-Evans, 1970, in correcting phenylbutazone half-life for height). In this way the majority of the variation remaining in SA excretion must be due to some factor other than urine pH.

Body weight and ASA metabolism

No correlation was found between body weight and excretion of SA (corrected for pH) or SUA. However, Cummings and Martin (1964) found a strong inverse relationship between plasma salicylate levels and body weight in the 10 healthy males studied. As these workers did not report any correlation between body weight and

urinary excretion of salicylate, and plasma levels of SA were measured after steady-state was reached following chronic dosing with 1 g of aspirin per dose, their study and the present work are not directly comparable.

Age and ASA metabolism

No correlation was found between age and excretion of SA (corrected for pH) or SUA. However, this finding differs from reports by Montgomery *et al.* (1986), Netter *et al.* (1986) and Ho *et al.* (1985). These studies showed that although there is no correlation between age and various pharmacokinetic parameters of SA, namely plasma clearance, maximal plasma concentration and AUC, significant positive correlations were found between age and the maximal plasma concentration and AUC of SUA. The effect of age on SUA is also supported by renal clearance data of Montgomery *et al.* (1986) and Ho *et al.* (1985). They found that renal clearance of SUA decreased with increasing age ($p < 0.001$), however they provide conflicting evidence for the renal clearance of SA. Ho *et al.* (1985) also showed that renal clearance of SUA correlated strongly ($p < 0.001$) with the renal clearance of creatinine. Therefore the decreased renal clearance and elevated plasma levels of SUA reported in the elderly are due to the age-related decrease in glomerular filtration rate (Kampmann *et al.*, 1979). However, despite the effects of age on plasma levels and renal clearance of SA and SUA, the 24-hour urinary recovery of these 2 metabolites was not found to be affected by age.

The lack of correlation between age and the 8-hour urinary

excretion of SUA found in the present study, is probably due to the comparatively limited age-range of 18 to 61 years. In the studies by Montgomery *et al.* (1986), Netter *et al.* (1985) and Ho *et al.* (1985), a higher age range was used where subjects up to 80 years of age participated in the studies. The necessity of increasing the age range to show a correlation between age and excretion of ASA metabolites was observed by Oldham (1983). In the lower age range of 21 to 65 years, no significant changes were observed between age and the 8-hour urinary excretion of aspirin metabolites, as confirmed in the present study. However, in the age range of 65 to 85 years, SA secretion was found to decrease with age ($p < 0.05$). Therefore, as no elderly people over 65 years participated in the present study, no age effect on salicylate metabolism was observed.

Sex differences and ASA metabolism

Sex differences in salicylate metabolism were found in the present study for females excreted 24% less SUA ($p < 0.01$) and 36% greater SA ($p < 0.01$) compared to males, in the 0 to 8-hour urine collection. Trnavska and Trnavsky (1983), Ho *et al.* (1985) and Miners *et al.* (1986) found comparable differences in plasma levels of the aspirin metabolites as the plasma concentration of SA was found to be significantly higher in females whilst SA plasma clearance was significantly lower. Miners *et al.* (1986) and Ho *et al.* (1985) also found significantly decreased renal clearance of SUA in females but no sex difference in renal clearance of SA. The sex difference in renal clearance of SUA is confirmed in the present study where females were found to excrete significantly

less SUA. However, the lack of sex difference in renal clearance of SA was not reflected in the present study, where significantly more SA was excreted in females. This discrepancy may be due to the values obtained for the renal clearance of SA not being adjusted for pH. Therefore any differences due to gender are probably being masked by the variation due to urine pH: a variation removed in the present study.

Trnavska and Trnavsky (1983) and Ho *et al.* (1985) also found an elevated aspirin AUC and elimination half-life in females, apparently due to an intrinsically lower activity of aspirin esterase in females.

Oral contraceptive steroids and ASA metabolism

Oral contraceptive steroids were found to have no effect on the 8-hour urine excretion of SA and SUA. However Gupta *et al.* (1982) found that salicylic acid AUC was significantly reduced in women who had used OCS for 2 months. Miners *et al.* (1986) supported this by finding that the half-life of SA was significantly decreased in OCS users compared to control females. The metabolic and renal clearance of SUA was also found to be significantly greater in OCS users. Therefore Miners *et al.* stated that the increased SA plasma clearance found in OCS users was due to increased glycine conjugation. However the present study does not support the results of Miners et al. and Gupta *et al.* as oral contraceptives were not found to induce the glycine conjugation of SA.

One reason for the contrasting results could be that the subjects

of Miners *et al.* were approaching basal conditions whereas the subjects of the present study represent a normal female population. Also, Miners *et al.* used 8 females in the OCS group and 8 in the control female group, whilst the present study 10 females used OCS and 45 were control females. These differences in the range and number of subjects used are reflected in the larger interindividual variations found in the present study.

Also Gupta *et al.* discovered that although plasma levels and the half-life of SA are decreased during short-term OCS use, during long-term use (of 2 years or greater) these factors return to the basal levels found in control females. As no details were taken concerning length of oral contraceptive use in the present study, differences in the length of OCS use may also account for the contrasting results.

Although Miners *et al.* and Gupta *et al.* have shown oral contraception to cause an increase in glycine conjugation of SA, the present work indicates that this indication cannot be observed when a normal relatively large population is observed. Therefore, the inductive effect of OCS on salicylate metabolism may be of little clinical importance.

Smoking and ASA metabolism

Smoking was found to have no effect upon ASA metabolism in the present study.

Cigarette smoke contains polycyclic hydrocarbons which are known to be potent enzyme inducers. Evidence has shown that polycyclic hydrocarbons are capable of increasing the activity of several

enzyme systems in liver microsomes, including N-demethylation, hydroxylation, reduction and glucuronide conjugation (Conney and Burns, 1962), although considerable substrate specificity is exerted. Smoking may have no effect upon the metabolism of SA, despite the constant exposure to polycyclic hydrocarbons, because the major metabolic pathway of SA, glycine conjugation, is non-microsomal. The enzyme system is situated in the hepatic mitochondria and polycyclic hydrocarbons appear to be potent **microsomal** enzyme inducers.

b) Genetic control of aspirin metabolism

Large interindividual differences in serum concentrations and elimination rate of SA and SUA have been reported and were also found in the present study. 0 to 13% of the given dose (600 mg ASA) was excreted as SA, with 6 to 96% of the dose excreted as SUA in the 8-hour urine collection. Similar ranges were also found by Caldwell *et al.* (1980) where 1 - 10% of the dose (900 mg ASA) was excreted as SA and 6 - 72% excreted as SUA in a 12-hour urine collection. Such large variations in salicylate levels have been reported by several workers. Levy and Hollister (1964) found salicylate half-life varied from 2.5 to 8.5 hours in the 17 healthy subjects studied. Similarly, Paulus *et al.* (1971) and Gupta *et al.* (1975) found 5-fold variations in serum SA levels in rheumatoid arthritis patients following chronic dosing with aspirin. Gupta *et al.* also studied the urinary excretion of ASA metabolites and established a strong correlation between maximum serum SA levels

and the urinary excretion of SUA. Therefore, they proposed that the interindividual differences in serum levels are determined, at least partly, by similar differences in maximum rates of SUA formation and excretion.

In the present study, observed SA excretion was found to be positively skewed. This deviation from a normal distribution indicates that one or more factors have a predominant control on SA excretion, which is to be expected as SA secretion is, at least partly, a function of pH. The distribution of corrected SA excretion was also found to be skewed but it was much nearer to a normal distribution than that of observed SA excretion, showing that the majority of the effect of urine pH on SA excretion had been removed. The distribution of SUA was also found to be skewed. Therefore corrected SA and SUA excretion are subject to polygenic and multifactorial control with one or more factors having a predominant influence. Significant sex differences in salicylate metabolism have been found in the present study and these could be one of the factors responsible for the skew distribution of corrected SA and SUA.

The metabolic ratio SA/SUA was determined for each individual as this is a further way of expressing the glycine conjugation capacity of an individual. It is a more sensitive assessment of SA metabolism when compared to the excretion of the major metabolite, SUA, as relatively more importance is given to the individual excreting smaller amounts of SUA. The distribution of the metabolic ratio was found to be strongly skewed and had no discrete sub-

groups but was a wide and continuous spread. The deviation from a normal distribution and the interindividual variation of the metabolic ratio are sufficiently large to indicate that there may be genetic control of SUA formation and excretion. However, as the distribution is not multi-modal and no discrete sub-groups found, genetic control cannot be isolated from environmental control.

In order to test the possibility of genetic control of salicylate metabolism further, a repeat study was performed in 13 subjects. 70% of the subjects tested excreted similar amounts of SUA in the 2 tests whereas only 15% of the subjects excreted similar amounts of corrected SA on each occasion, and hence only 15% of the subjects had similar metabolic ratios for each occasion. Although SUA excretion was found to be largely reproducible, large intra-individual variations of 25 - 60% exist in 30% of the subjects. Also, the reproducibility of corrected SA excretion and the metabolic ratio was very poor with large intra-individual variations of 25 to 400% and 25 to 500% respectively occurring in 85% of the subjects. Plus, although the time difference between repeat tests varied from 7 to 27 months, it appeared to have no effect upon the reproducibility obtained.

Therefore, although the wide, skewed distribution obtained for the metabolic ratio indicated that there may be genetic control of SA metabolism, the lack of reproducibility shows that any genetic control present must be comparatively weak. Hence although genetic control may be partially responsible for the skewed distribution found for glycine conjugation of SA, further environmental factors

may also be responsible for this deviation from a normal distribution. The lack of reproducibility further indicates that environmental factors may have a stronger influence on salicylate metabolism. The reproducibility results of SUA found in the present study, are supported by Levy and Hollister (1964) where serum elimination rate constants of SA were found to differ by a maximum of 20% in 70% of subjects studied (12 out of 17) in repeat tests. When these results are combined with the strong correlation between maximum serum SA levels and SUA urine excretion (as found by Gupta *et al.*, 1975), they provide similar values of reproducibility to those obtained in the present study.

Large variations in serum SA levels and the reproducibility of serum levels of SA indicate that there may be genetic control of SA metabolism. However, when Evans and Clarke (1961) studied the possibility of polymorphism of salicylate metabolism, serum levels determined 3 hours after ingestion of sodium salicylate showed a normal distribution. Therefore, despite the large variation, Evans and Clarke could detect no polymorphism of serum salicylate levels. The lack of clear genetic control was also found by Caldwell *et al.* (1980) and Hutt *et al.* (1986). In these population studies (n = 85 and n = 129 respectively) a unimodal distribution of SUA excretion was found, with the distribution presented by Hutt *et al.* appearing slightly skewed (although the probit plot showed a normal distribution) Hutt *et al.* also showed a skewed distribution for SA excretion, similar to that obtained in the present study for observed SA excretion, and so the deviation from a normal distribution is probably mainly due to urine pH. Caldwell *et al.*

performed reproducibility studies on 6 of their subjects and they found that the **relative** extent of glycine conjugation (that is, SUA excretion being expressed as a percentage of the total salicylate excreted) only varied by a maximum of 12%. The discrepancy between these reproducibility results and the results of the present study are due to relative amounts being expressed in the former and absolute amounts expressed in the latter. Caldwell *et al.* stated that total recovery of SA (and therefore recovery of the major metabolite, SUA) varied, but the extent of variation was not reported.

The population studies performed on serum SA levels (Evans and Clarke, 1961) and urinary salicylate metabolites (Caldwell *et al.*, 1980; Hutt *et al.*, 1986) all failed to show polymorphism of salicylate metabolism. The lack of clear genetic control was also confirmed in the present study.

However, Furst *et al.* (1977) did find some indication of genetic control when studying salicylate metabolism in fraternal and identical twins. Intrapair variation in maximum serum SA levels and plasma clearance of SA were significantly less for identical twins than for fraternal twins in both acute IV and chronic oral dosing. Intrapair variation in SUA excretion rate and V_{max} was significantly less in identical twins on chronic oral dosing alone. Hence, this study shows that there may be some genetic control of aspirin metabolism, as determined from both plasma and urinary data. It must be noted that the subjects taking part in the study of Furst *et al.* were hospitalised prior to the study and so environmental variation was kept to a minimum, unlike the

population studies where normal day-to-day routine was not interrupted.

Further support for possible genetic control of ASA metabolism is given by Emudianughe *et al.* (1986). A population study of 122 Nigerians was performed and wide variations in SUA and salicylic acid glucuronide (SAG) excretion were found. The SUA/SAG ratio was found to be significantly higher in Caucasians than in black Nigerians, suggesting that Caucasians have a higher capacity for glycine conjugation. Such a racial difference in turn implies genetic control.

Despite the work by Furst *et al.* (1977) and Emudianughe *et al.* (1986), no firm evidence has been provided for the genetic control of aspirin metabolism, as various population studies, including the present work, have failed to show any polymorphism. This is probably due to a number of other factors which affect aspirin metabolism and which mask any genetic control that may be present. In the present study, sex has been found to have a significant effect on ASA metabolism although there is conflicting evidence concerning the effect of age, body weight and oral contraceptive steroids. Recent reports have shown that fasting or not fasting prior to aspirin ingestion had no effect upon aspirin bioavailability (Koch *et al.*, 1986) and caffeine intake, although increasing aspirin bioavailability, had no effect upon salicylate excretion (Yoovathaworn *et al.*, 1986).

Therefore, age (if greater than 65), sex and urine pH are the only factors known to affect SA metabolism. Further investigation must

be performed in order to determine further causes of the wide inter- individual variation of salicylate metabolism.

CHAPTER FOUR

PARACETAMOL METABOLISM IN HUMAN VOLUNTEERS

4.1 INTRODUCTION

Several studies have been performed in order to determine the effects of various environmental factors on paracetamol metabolism (including Mucklow *et al.*, 1980; Miners *et al.*, 1983). However, few workers have studied the possibility of genetic control of paracetamol metabolism. In the present study a therapeutic dose of paracetamol was given to a large number of healthy adult volunteers and the urinary excretion of free paracetamol (P) and its major metabolites; paracetamol glucuronide (PG) and paracetamol sulphate (PS) were determined. In this way the effects of various environmental factors on paracetamol metabolism were determined and the possibility of genetic control investigated.

4.2 PROCEDURE

Paracetamol (1 g) was taken orally in the morning by 99 volunteers, 68 male and 31 female, after first voiding their bladders. An 0 - 8 hr urine collection was obtained and the urinary metabolites paracetamol (P), paracetamol glucuronide (PG) and paracetamol sulphate (PS) were assayed by HPLC as described in Chapter 2.8a. The quantity of PG and PS excreted is expressed as the equivalent weight of P, using the correction factors given in Chapter 2.12.

The age range for males was 18 to 60 years with a mean age of

36.5 ± 1.4 years (mean ± SE) and the weight range was 48 to 102 kg with a mean weight of 74.9 ± 1.4 kg (mean ± SE). The age range for females was 19 to 57 years with a mean age of 34.5 ± 1.9 years and the weight range was 47 to 95 kg with a mean weight of 60.6 ± 2.1 kg.

The Student's t test was used as the test for statistical significance throughout this study.

4.3 RESULTS

a) Environmental control of paracetamol metabolism

Table 4.1 gives the total amount and percentage dose excreted in 8 hours of P, PG and PS, following a therapeutic dose of 1 g of paracetamol. 21.0 ± 0.9 mg (2.1 ± 0.1%) of free paracetamol was excreted, together with 427.9 ± 11.1 mg (42.8 ± 1.1%) of PG and 217.5 ± 8.6 mg (21.75 ± 0.9%) of PS. In total, 660.9 ± 15.5 mg (66.1 ± 1.55%) of the dose was recovered in the 8-hour urine. Individual results (expressed in mg) together with the sex, age, weight, smoking habits and concomitant drug treatment of each volunteer are given in Appendix 2.

Figs. 4.1 and 4.2 show the relation between the 8-hour urinary excretion of the 2 major paracetamol metabolites, PG and PS. No correlation was found between the total amounts of PG and PS excreted ($r = 0.102$, $p > 0.1$, Fig. 4.1). However, when PG and PS were expressed as the percentage of dose recovered, a strong negative correlation was found between the two metabolites ($r = 0.924$ $p < 0.01$, Fig. 4.2).

FIGURE 4.1

Relationship between the urinary excretion of PG and PS
(expressed as the total amount recovered)

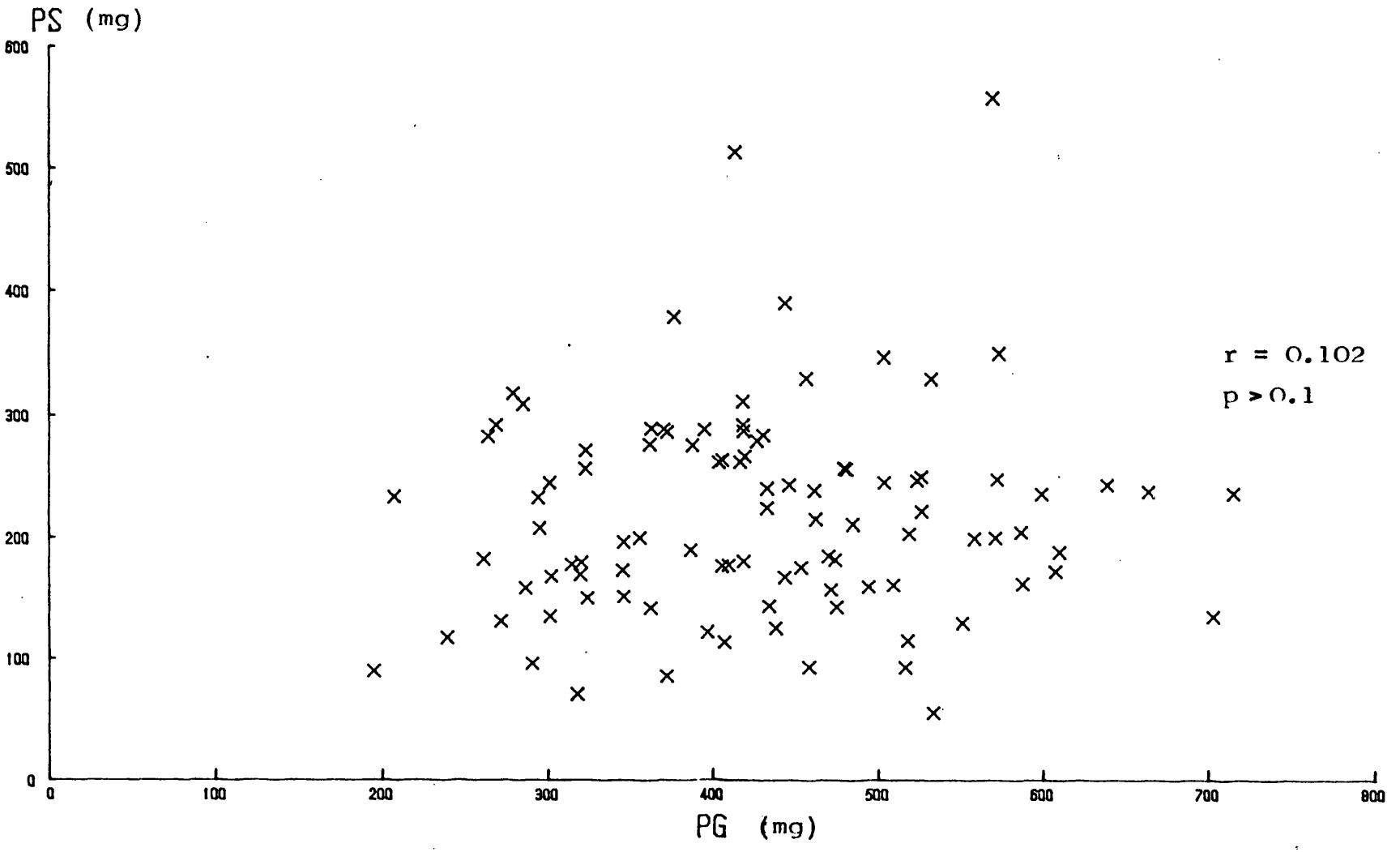


FIGURE 4.2

The relationship between the urinary excretion of PG and PS, expressed as the % of dose recovered.

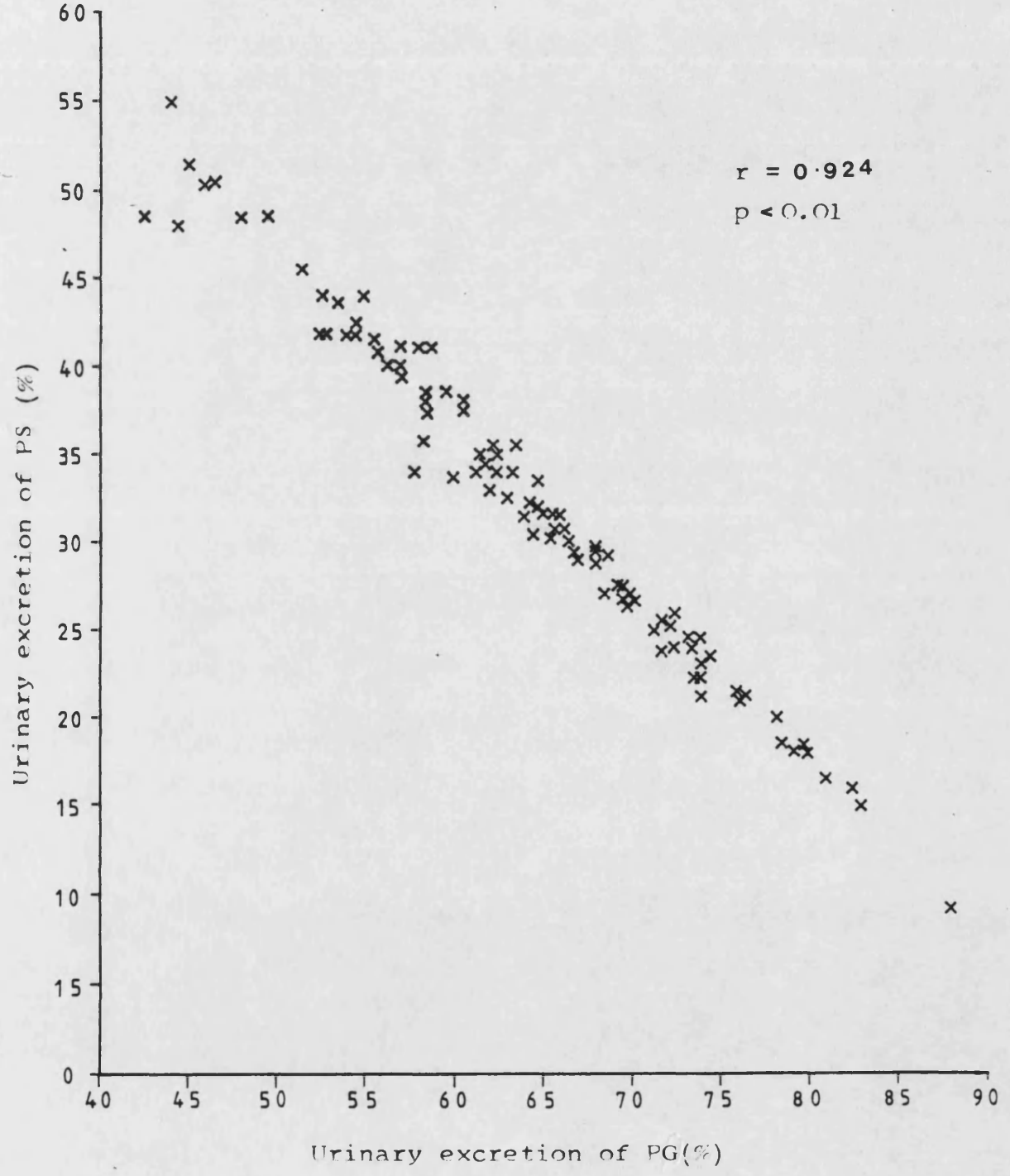


Table 4.1. Urinary recovery (0-8 hr) of paracetamol and its metabolites following therapeutic dosage in 99 normal volunteers.

Paracetamol metabolite	Total excreted mean ± SE (mg)	% Dose excreted mean ± SE (%)	Range (mg)
PG	427.9 ± 11.1	42.79 ± 1.11	194.8 - 715.4
PS	217.5 ± 8.6	21.75 ± 0.86	71.3 - 560.5
P	21.0 ± 0.9	2.10 ± 0.09	3.7 - 60.5
PG + PS + P	660.9 ± 15.5	66.09 ± 1.55	268.1 - 1152.0

Effect of age and weight

Urinary excretion of P, PG and PS all failed to show a correlation with age ($r = 0.066, -0.260, 0.202$ respectively, $p > 0.1$). Similarly, excretion of P, PG and PS also failed to show a correlation with body weight ($r = 0.004, -0.082, 0.007$ respectively; $p > 0.1$). The relationships between age and weight and the urinary excretion of P, PG and PS are shown in Figs. 4.3 to 4.5.

Effect of gender

The urinary excretion of the paracetamol metabolites, P, PG and PS was compared in males and females. No significant difference was found between the sexes for any of the metabolites as shown in Table 4.2.

FIGURE 4.3a

Effect of age on the 0-8 hr urinary excretion of free paracetamol

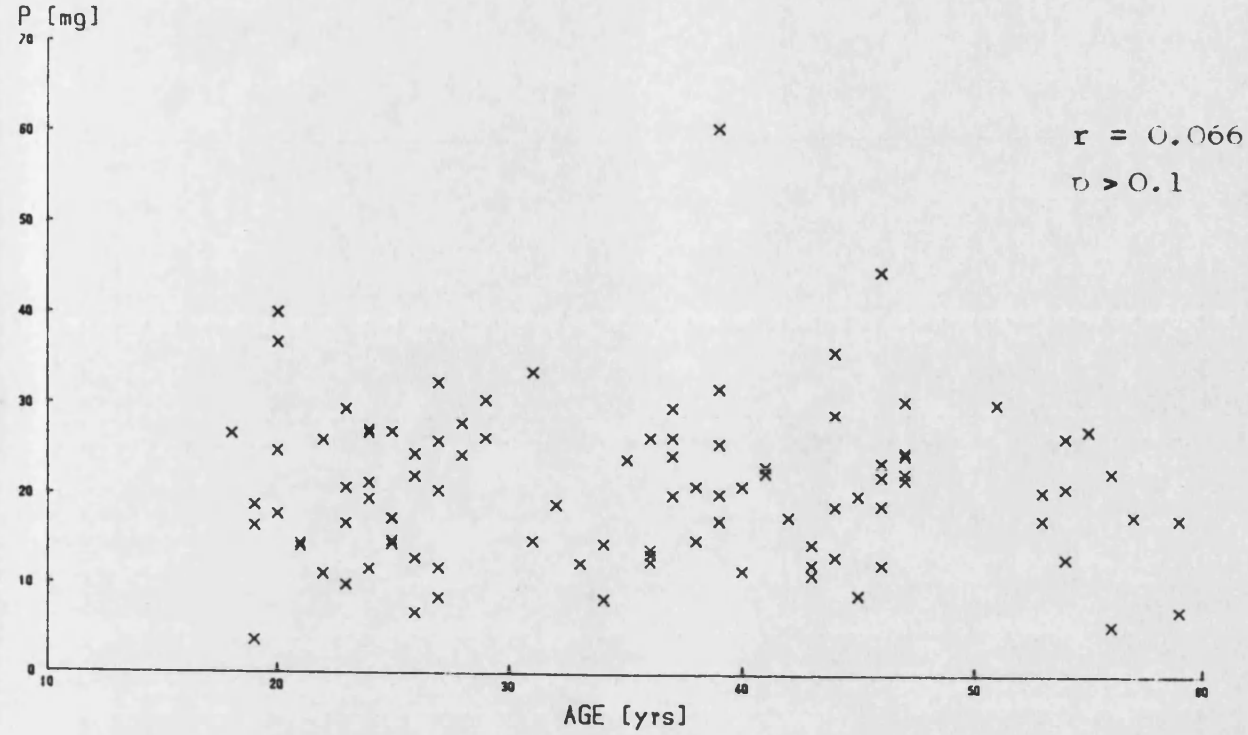


FIGURE 4.3b

Effect of body weight on the 0-8 hr urinary excretion of free paracetamol.

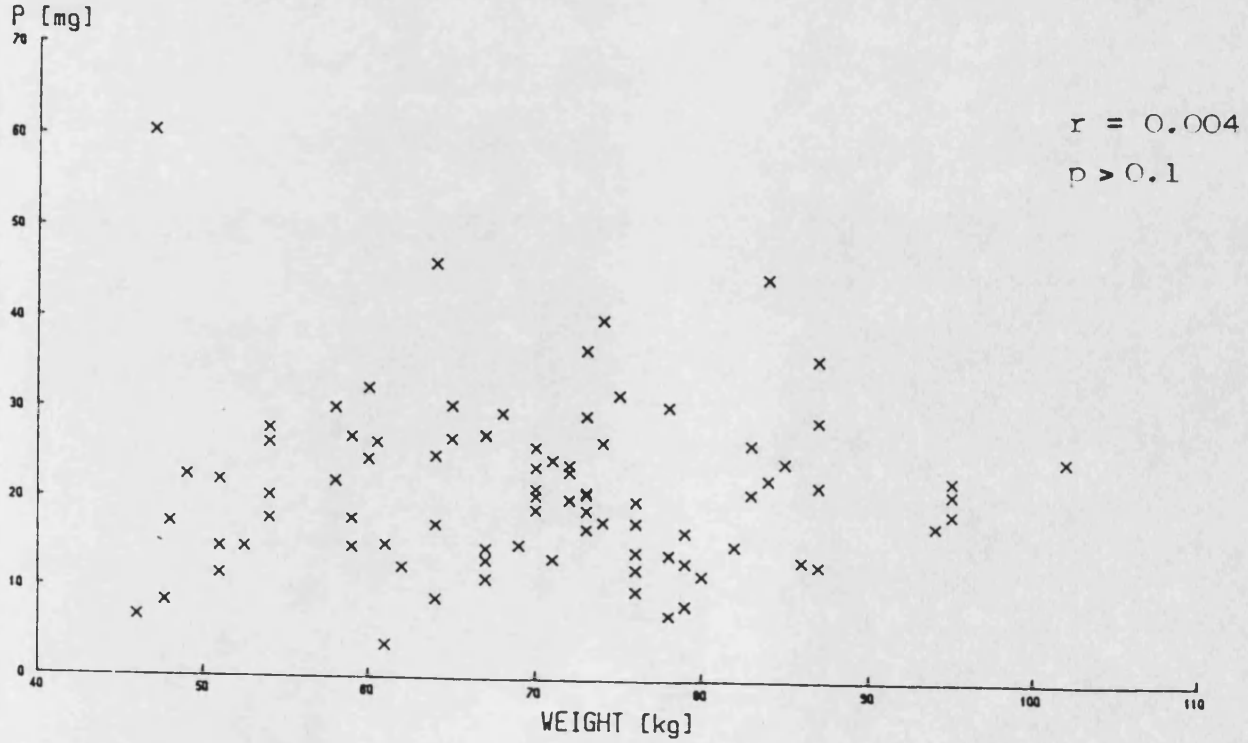


FIGURE 4.4a

Effect of age on the o-8 hr urinary excretion of PG

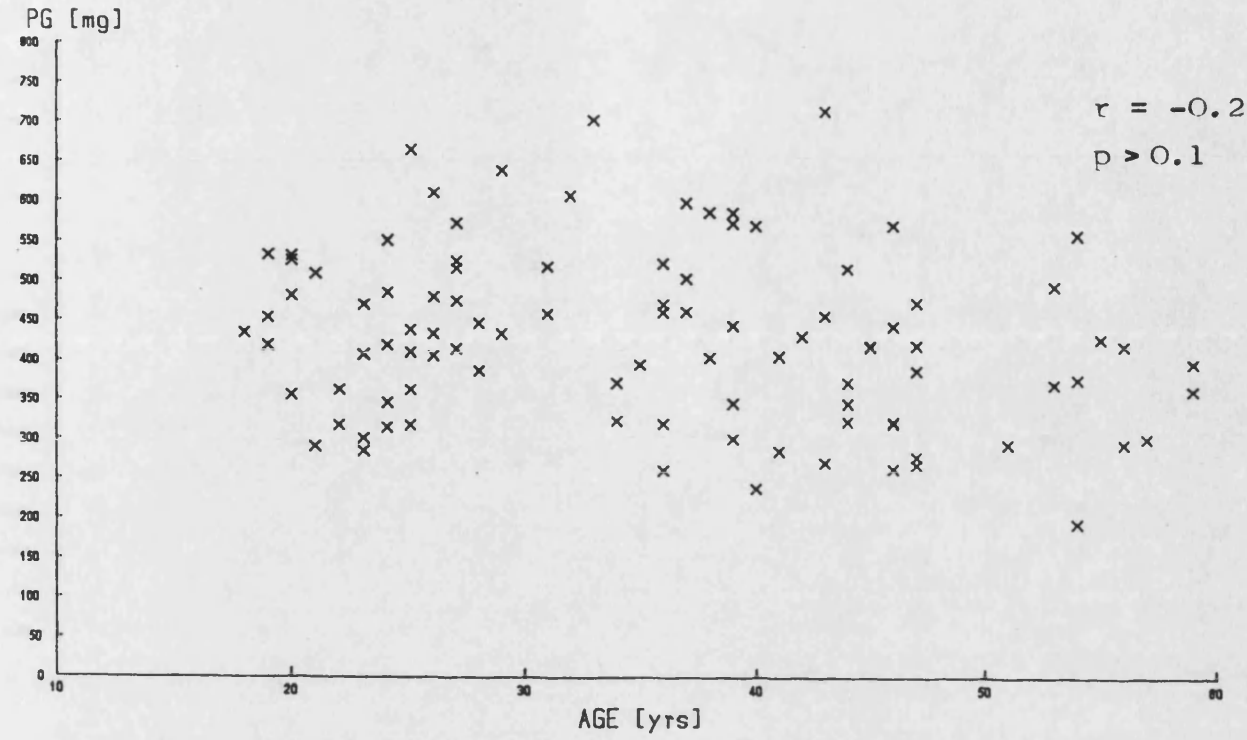


FIGURE 4.4b

Effect of bodyweight on the 0-8 hr urinary excretion of PG

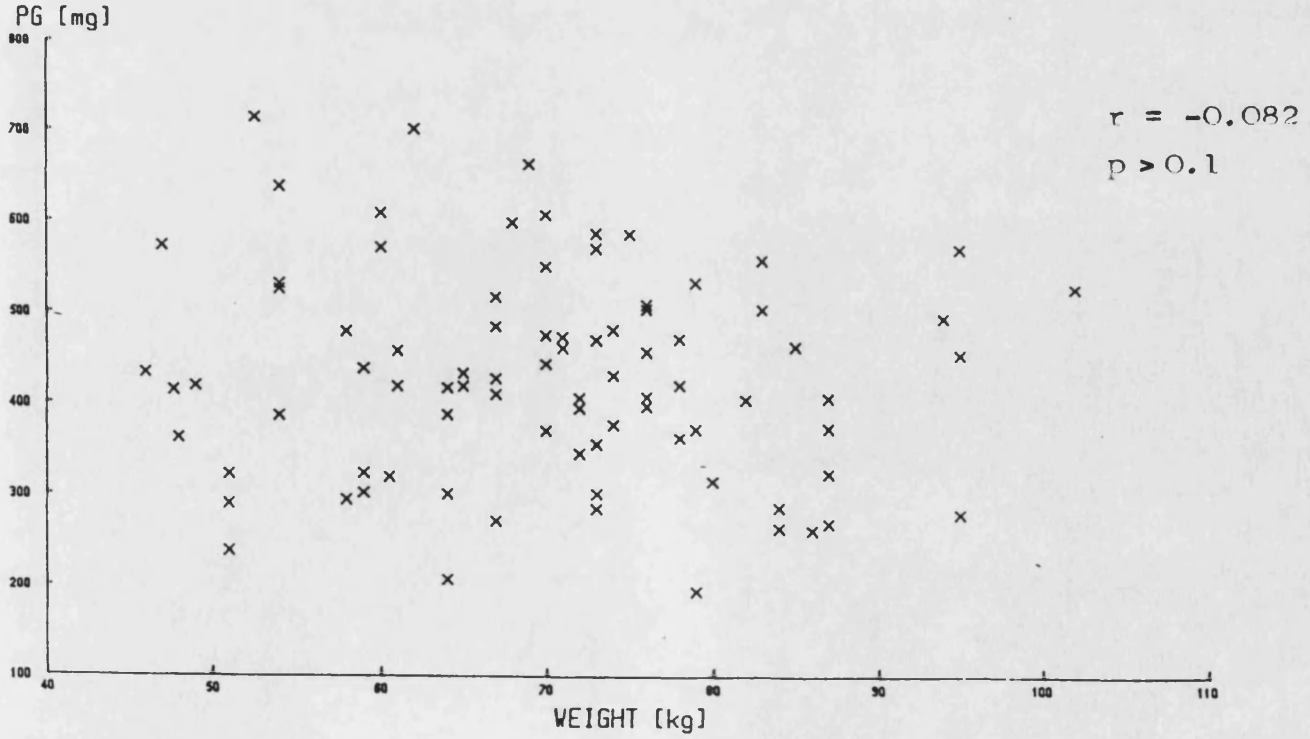


FIGURE 4.5a

Effect of age on the 0-8 hr urinary excretion of PS.

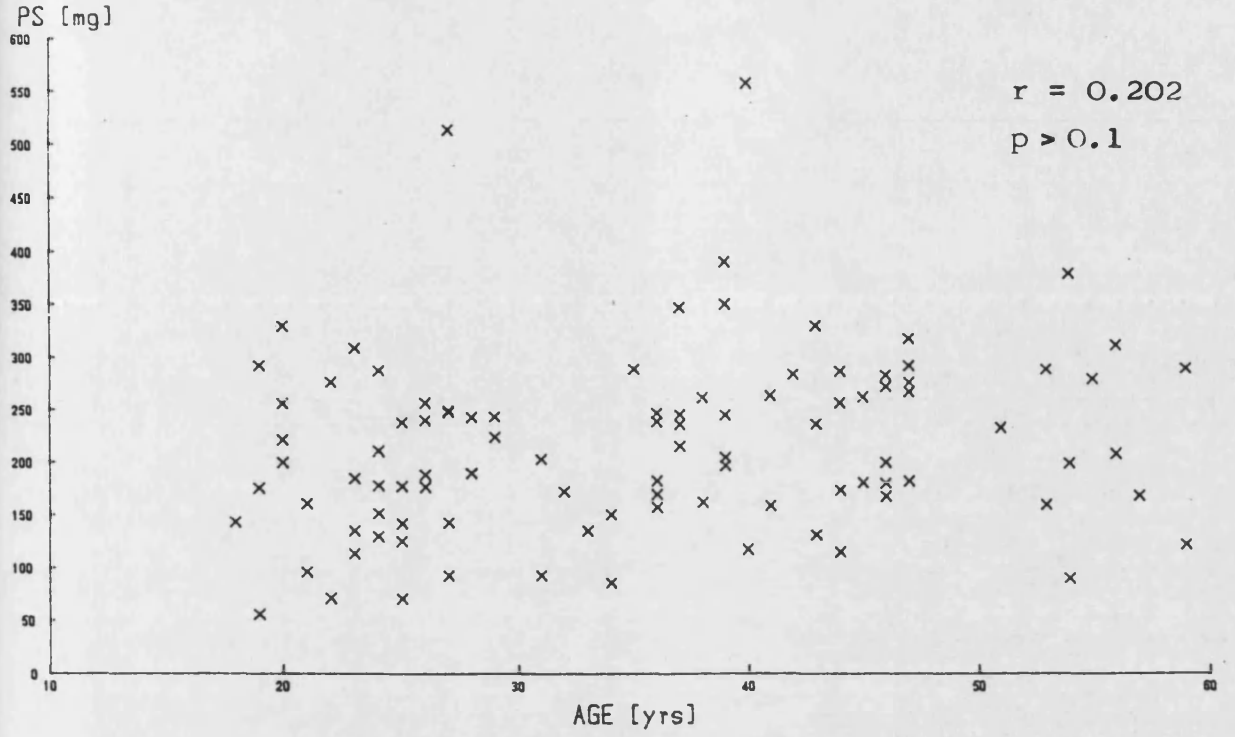


FIGURE 4.5b

Effect of body weight on the 0-8 hr urinary excretion of PS.

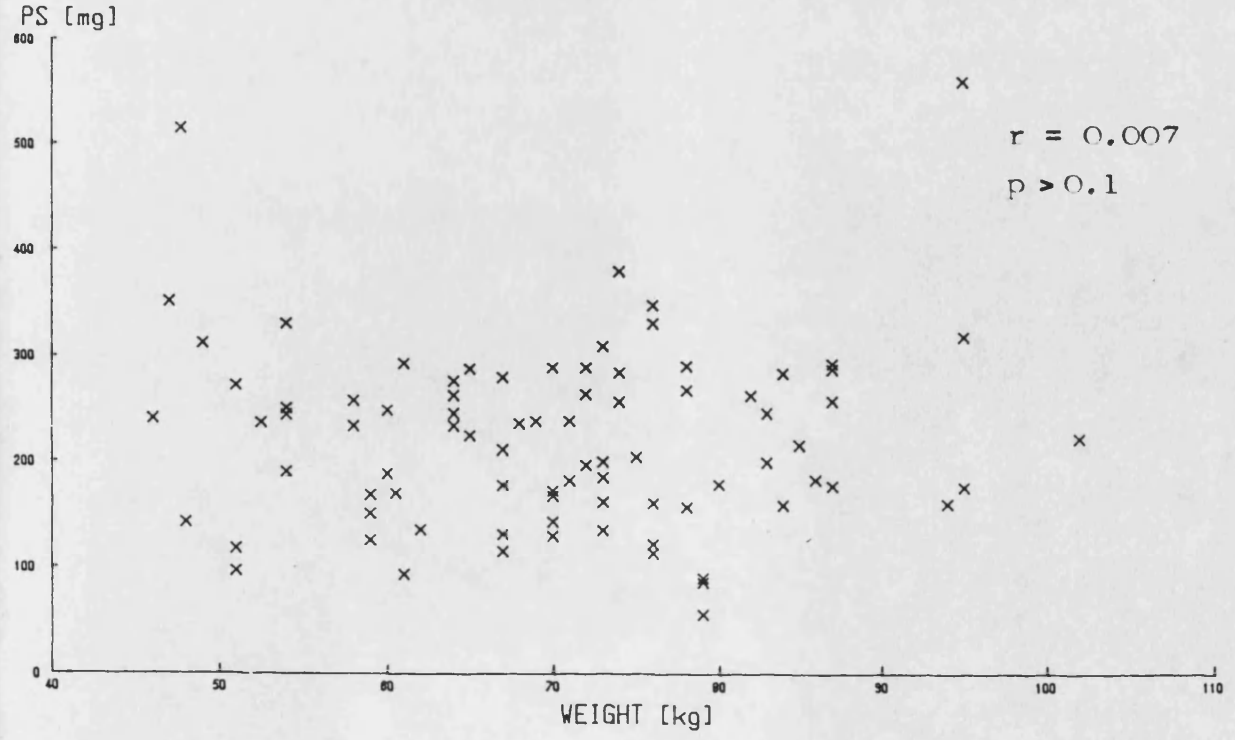


Table 4.2. Urinary excretion (0 - 8 hr) of paracetamol and its metabolites (mean ± SE) in males and females.

Paracetamol metabolite	Male (n = 68) (mg)	Female (n = 31) (mg)
PG	414.0 ± 12.7	449.0 ± 21.8
PS	212.0 ± 9.0	229.5 ± 19.3
P	21.6 ± 1.0	19.7 ± 1.9

Effects of smoking

The effect of smoking on urinary excretion of paracetamol and its metabolites was assessed. Male smokers and male non-smokers showed no significant difference in the urinary excretion of P, PG and PS. Female smokers also showed no significant difference in the urinary excretion of P and PS compared to female non-smokers. However, female smokers were found to excrete significantly more PG than female non-smokers. These results are shown in Table 4.3 and Fig. 4.6.

FIGURE 4.6

Effect of smoking on the 0-8 hr urinary excretion of paracetamol and its major metabolites, PG and PS in males and females.

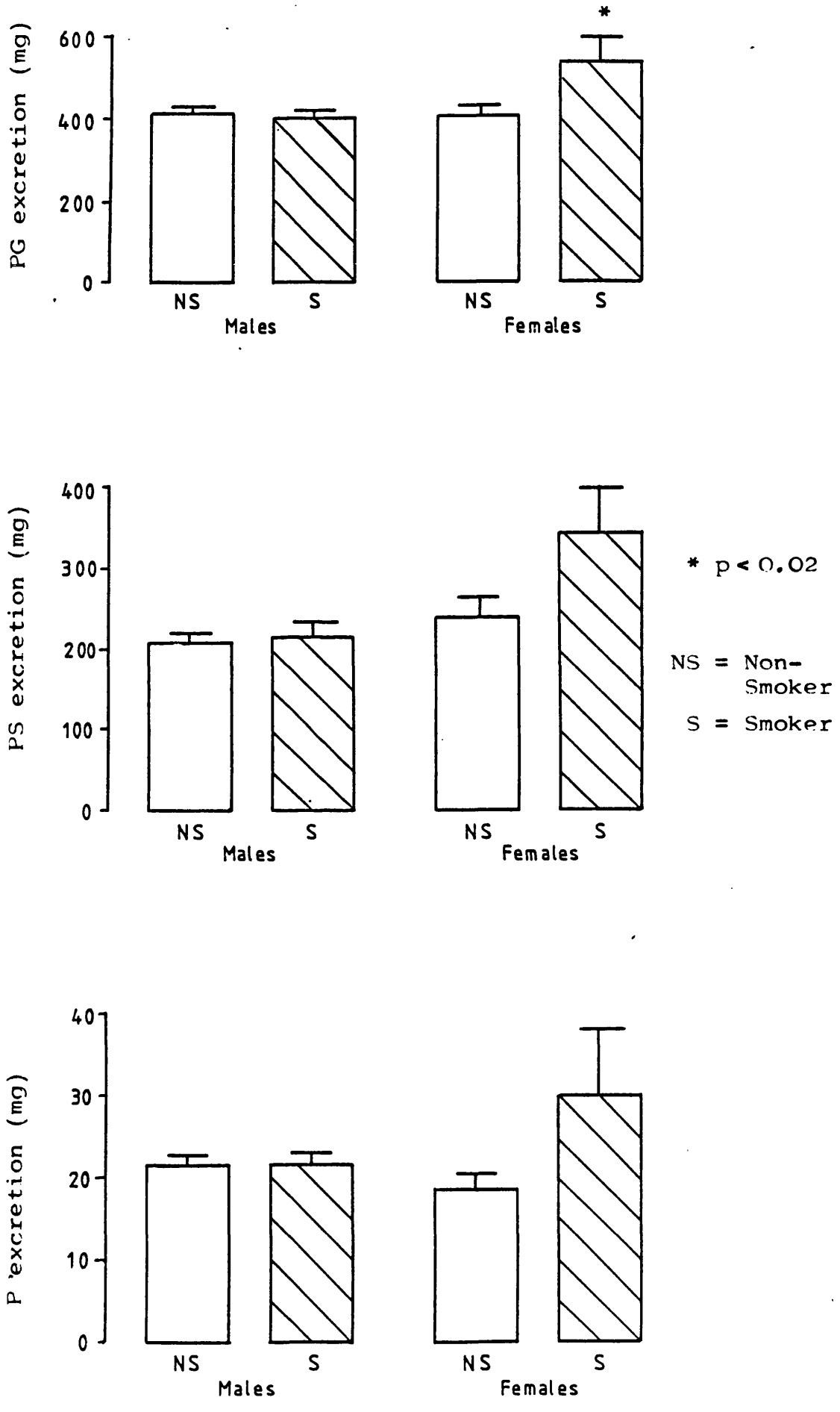


Table 4.3. Urinary excretion of paracetamol and its metabolites (mean ± SE) in smokers and non-smokers.

Paracetamol metabolite	MALE Non-smoker (n=49)	MALE Smoker (n=18)	FEMALE Non-smoker (n=15)	FEMALE Smoker (n=5)
PG	414.8 ± 15.2	401.1 ± 22.0	*406.6 ± 22.9	539.7 ± 55.6
PS	209.8 ± 10.4	216.2 ± 19.6	240.7 ± 23.1	345.8 ± 57.1
P	21.5 ± 1.2	21.5 ± 2.0	18.5 ± 2.0	29.8 ± 8.1

* $p < 0.02$ when compared to smoker.

N.B. The female smokers and non-smokers are all control females. i.e. None of the females in the above table (Table 4.3) use oral contraceptives.

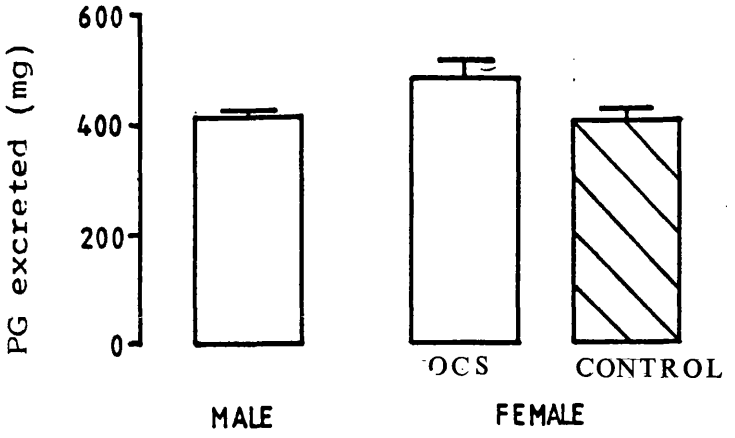
Effect of oral contraceptive steroids

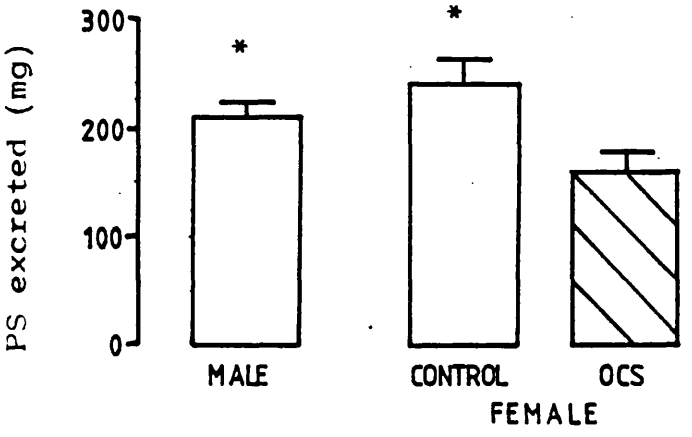
In order to study the effects of oral contraception on paracetamol metabolism, the data obtained from OC users (all non-smokers) was compared with males and non-smoking females (due to the significant effect of smoking on paracetamol metabolism in females).

Oral contraceptive steroid (OCS) users were found to excrete more PG than males and control females but the differences just failed to reach significance ($p > 0.05$). Conversely, OCS users excreted significantly less PS than both males and control females ($p < 0.02$). No significant difference was found in P excretion between any of the 3 groups studied ($p > 0.1$). These differences are shown in Table 4.4 and Fig. 4.7.

FIGURE 4.7

Urinary excretion of paracetamol and its major metabolites in males, females using OCS and females not using OCS.





* $p < 0.02$ compared to OCS users

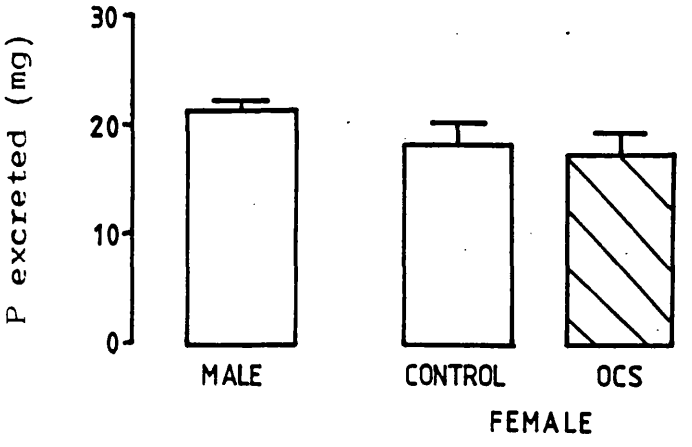


Table 4.4. Urinary excretion of paracetamol and its metabolites (mean ± SE) in males, females using oral contraceptive steroids (OCS users) and females not using OCS.

Paracetamol metabolite	Males (n=68)	OCS users (n=11)	Control females (n=15)
PG	414.0 ± 12.7	483.9 ± 38.2	406.6 ± 22.9
PS	*212.0 ± 9.0	161.6 ± 17.7	*240.7 ± 23.1
P	21.6 ± 1.0	17.4 ± 2.3	18.5 ± 2.0

* $p < 0.02$ when compared to OCS users.

b) Genetic control of paracetamol metabolism

Figures 4.8 to 4.10 show the frequency distribution histograms and probit plots for the urinary excretion of unchanged paracetamol, PG and PS respectively. The linear probit plots for P and PG excretion (x^2 = 19.4, 13.3 respectively; $p > 0.1$) together with their frequency distribution histograms, show a normal distribution for these 2 urinary metabolites. However, the frequency distribution of PS excretion appears to be slightly skewed, with the deviation from a normal distribution being supported by the non-linear probit plot (x^2 = 26.5, $p < 0.01$).

The metabolic ratios were then determined for each individual to further assess the glucuronidation and sulphation capacities of the subjects and these were calculated as shown below:

FIGURE 4.8

Distribution of the 0-8 hr urinary excretion of unchanged paracetamol in 99 volunteers.

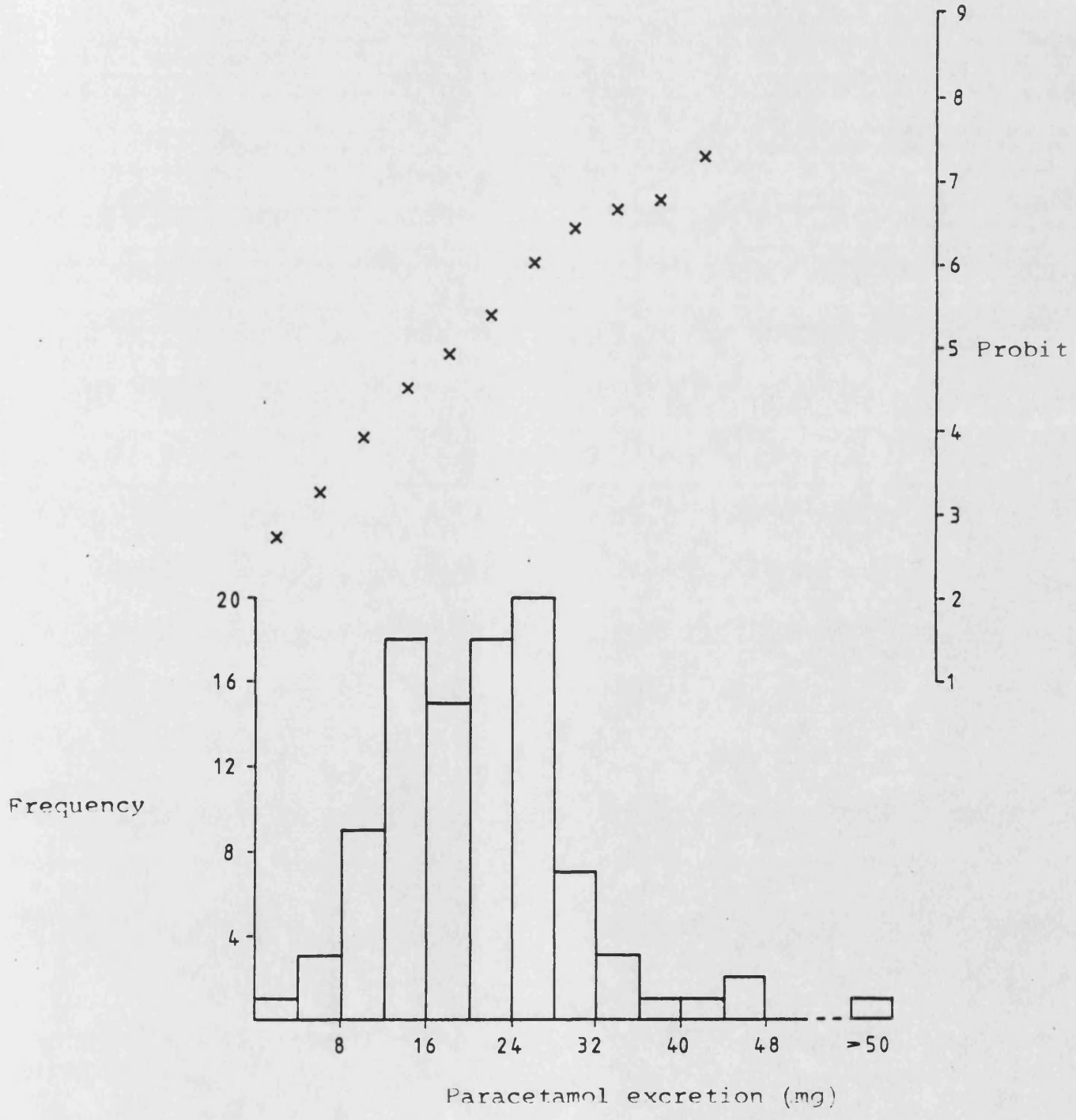


FIGURE 4.9

Distribution in the 0-8 hr urinary excretion of PG in 99 volunteers.

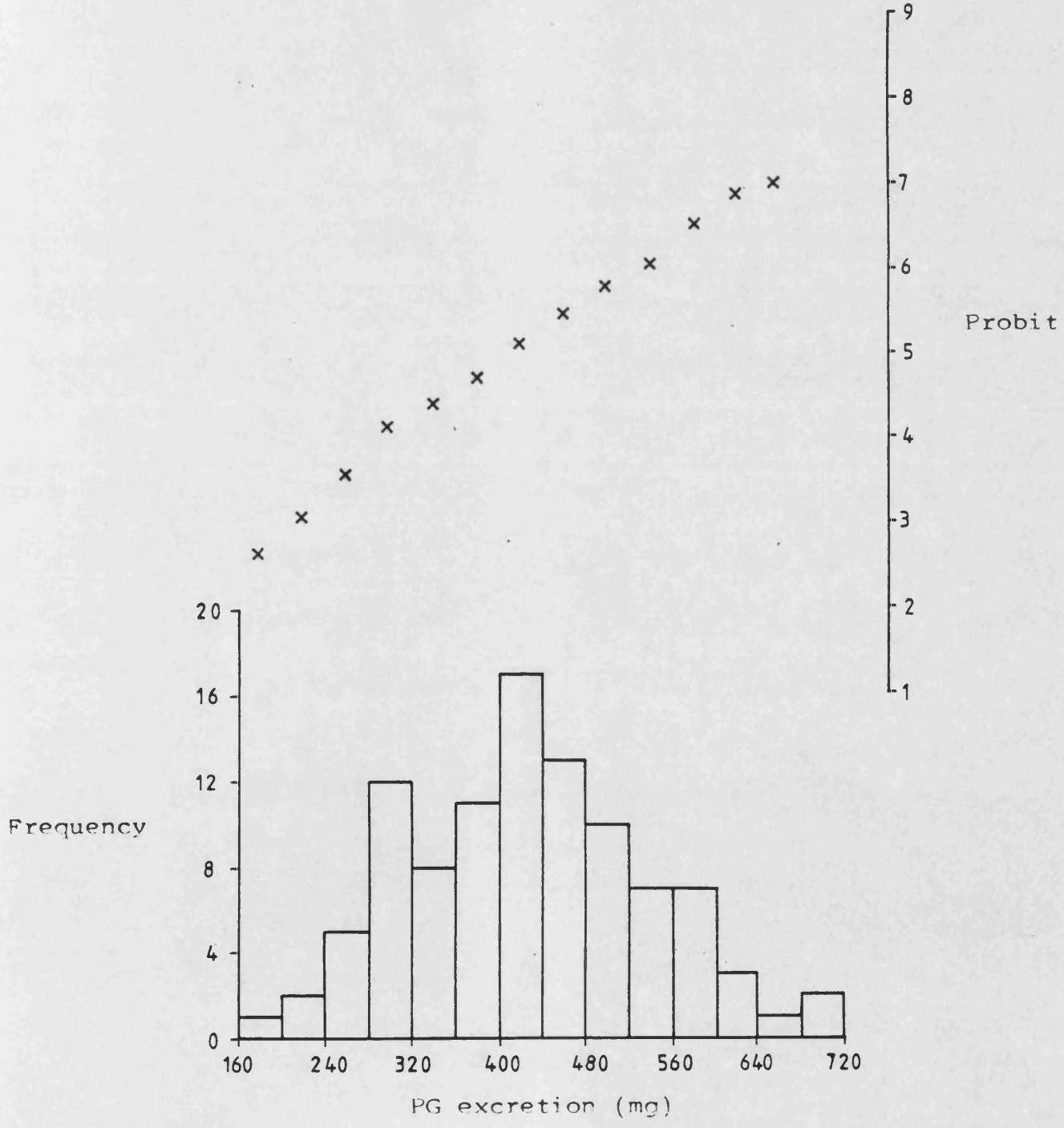
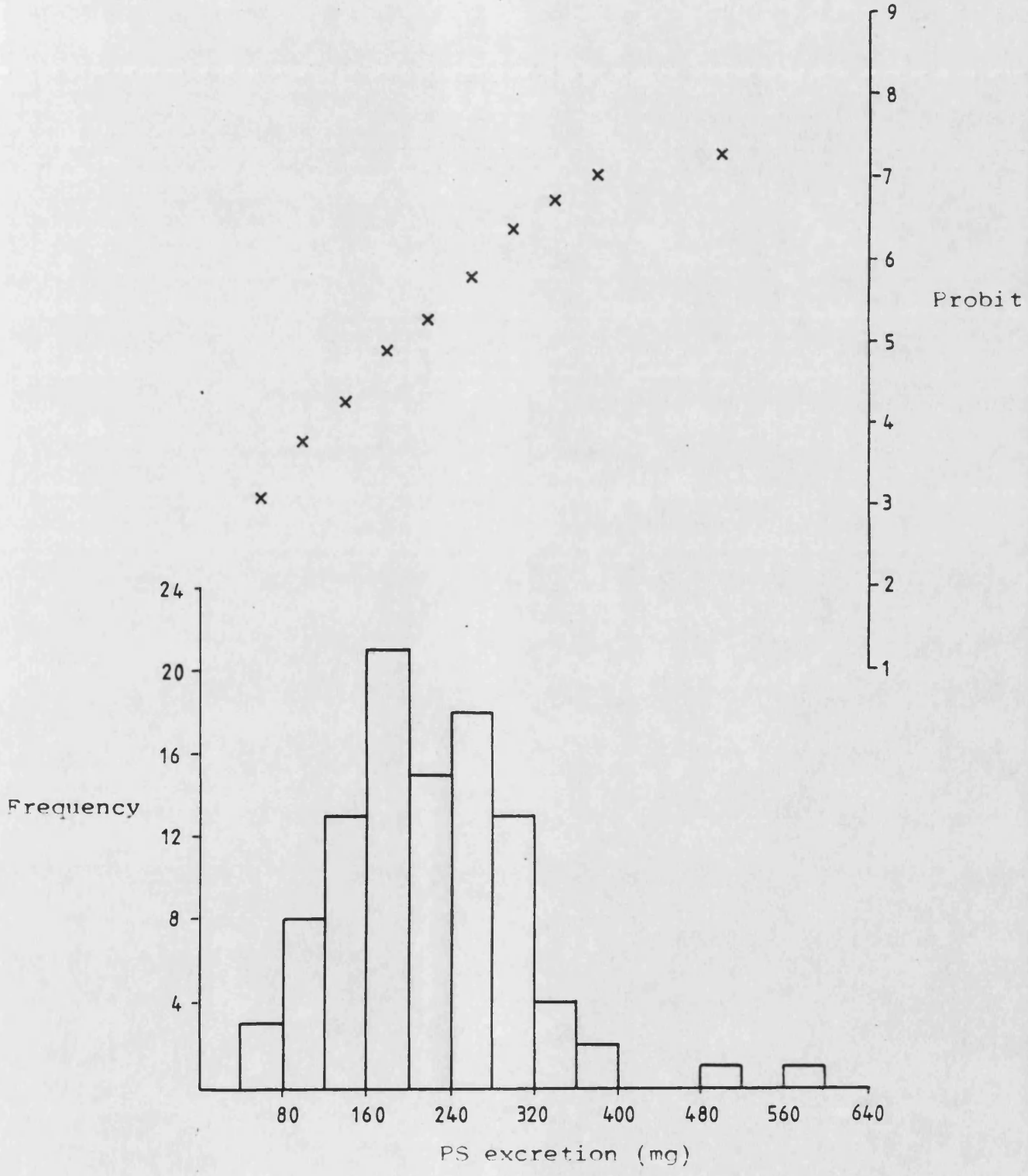


FIGURE 4.10

Distribution of the 0-8 hr urinary excretion of PS in 99 volunteers.



$$\text{Metabolic Ratio; P/PG (Glucuronidation Ratio)} = \frac{\text{Total amount of unchanged P excreted (mg)}}{\text{Total amount of PG excreted (mg)}}$$

$$\text{Metabolic Ratio; P/S (Sulphation Ratio)} = \frac{\text{Total amount of unchanged P excreted (mg)}}{\text{Total amount of PS excreted (mg)}}$$

The frequency distribution histograms and probit plots for the metbolic ratios, P/PG and P/PS are shown in Figures 4.11a and 4.11b. The histograms for both ratios are normally distributed, with the linear probit plots (x^2 = 18.1 for P/PG; $p > 0.1$ and x^2 = 9.1 for P/PS; $p > 0.1$) confirming the normal distribution. A further metabolic ratio, PG/PS was calculated in order to study the relative capacities of glucuronidation and sulphation for each individual.

$$\text{Metabolic Ratio, PG/PS (Relative conjugation ratio)} = \frac{\text{Total amount of PG excreted (mg)}}{\text{Total amount of PS excreted (mg)}}$$

The frequency distribution histogram given in Figure 4.12 appears strongly skewed, and the non-linear probit plot (x^2 = 20.6, $p < 0.05$) supports the deviation from a normal distribution.

FIGURE 4.11a

Distribution for the metabolic ratio $\frac{P}{PG}$ in 99 Volunteers.

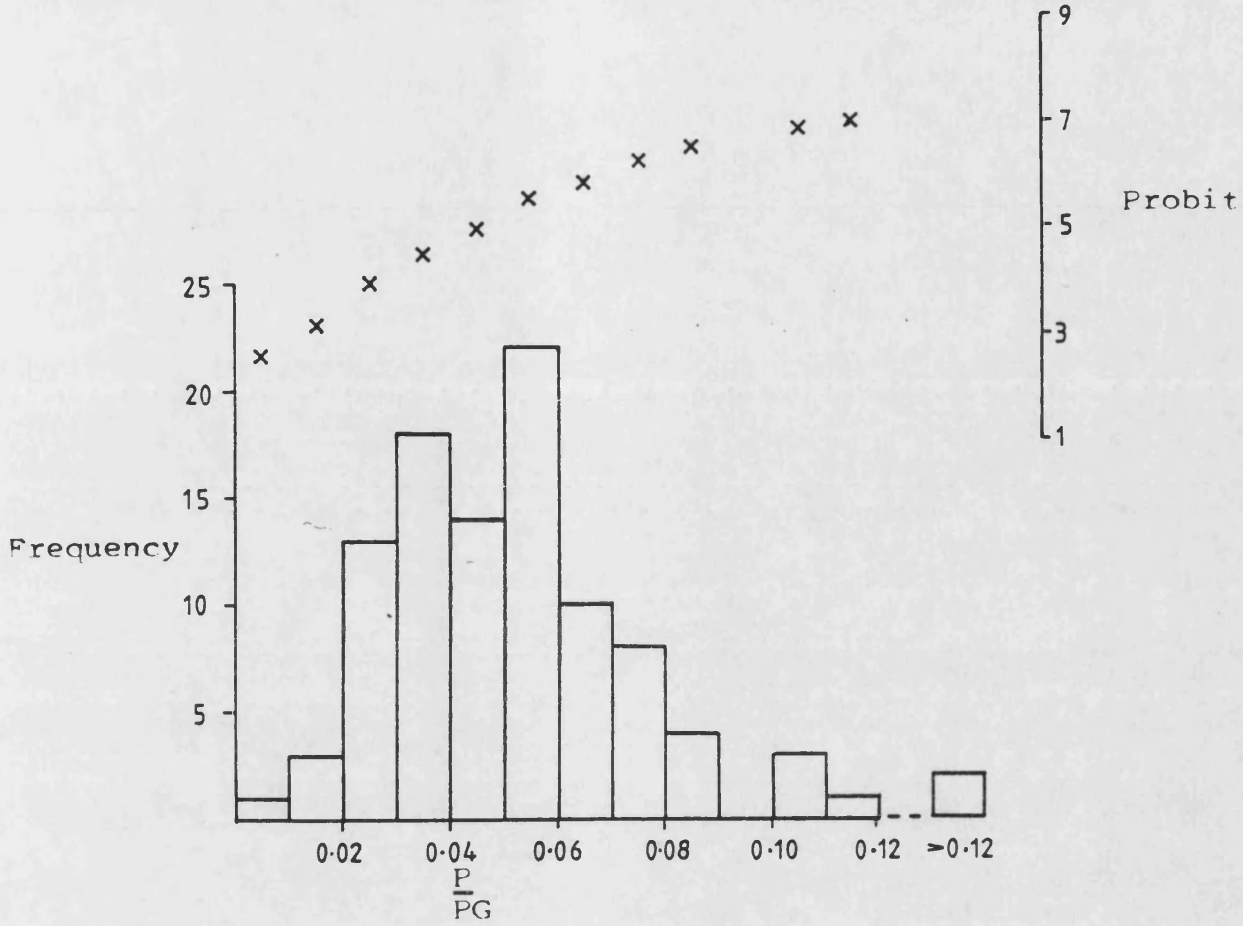


FIGURE 4.11b

Distribution for the metabolic ratio $\frac{P}{PS}$ in 99 volunteers.

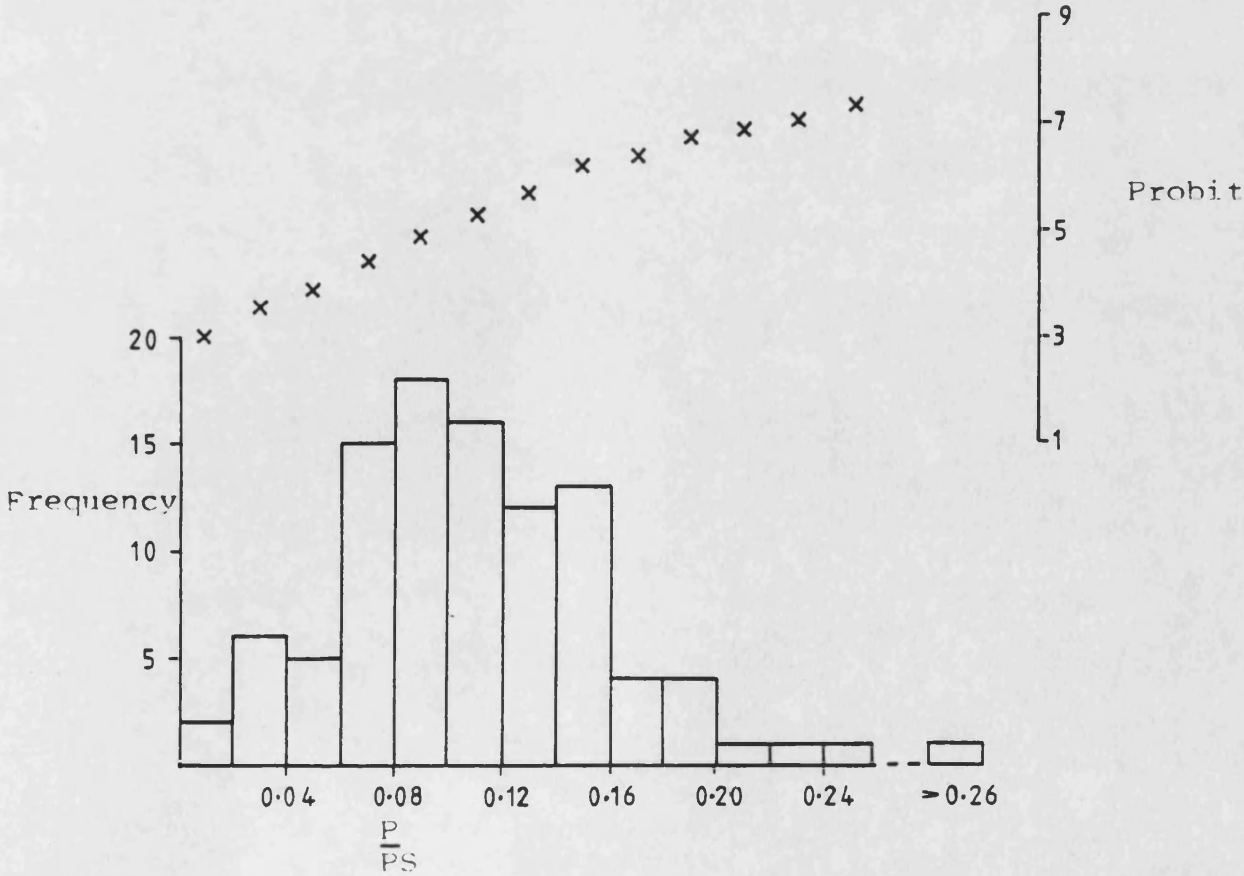
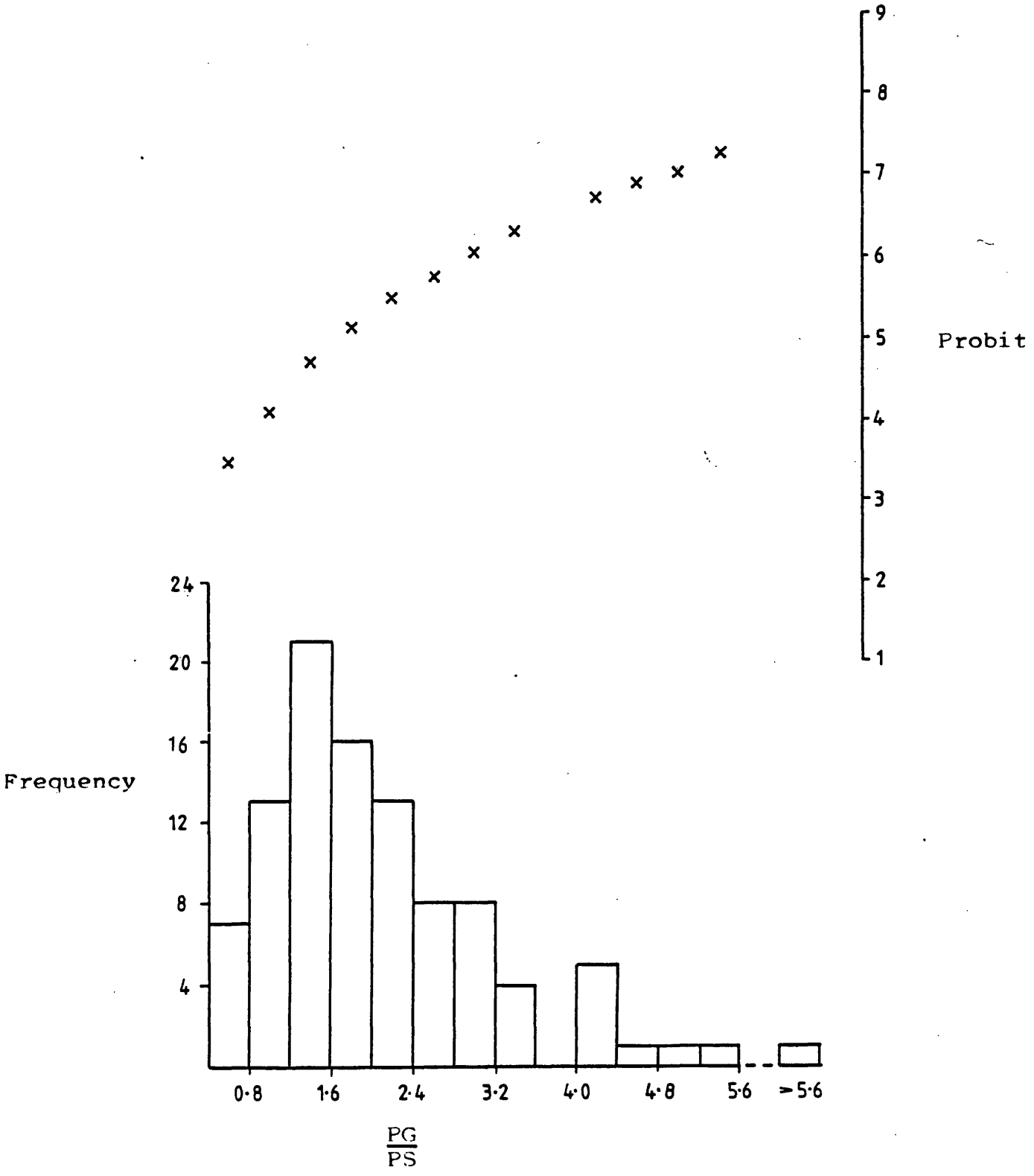


FIGURE 4.12

Distribution for the metabolic ratio $\frac{PG}{PS}$ in 99 volunteers.



4.4 DISCUSSION

a) Environmental control of paracetamol metabolism

Following oral administration of 1 g of paracetamol to 99 healthy volunteers, the 0 - 8 hour urinary excretion of P, PG and PS was determined. PG and PS were found to be major metabolites of paracetamol, with 42.8 ± 1.1% of the dose excreted as PG, 21.75 ± 0.9% excreted as PS and 2.1 ± 0.1% excreted as unchanged P. In total, 66.1 ± 1.55% of the dose was recovered as P + PG + PS in the 0 - 8 hour urine. These results are similar to those of Cummings *et al.* (1967) and Potter *et al.* (1974), who found similar urinary excretions for paracetamol and its 2 major metabolites, PG and PS.

As PG and PS are the 2 major paracetamol metabolites, the relationship between the two was studied in the present work. No correlation was found between the total urinary excretion (expressed in mg) of PG and PS. However, there were large interindividual variations found in the urinary excretion of PG and PS which may have masked possible intraindividual correlation between the metabolites. Thus, the urinary excretion of both PG and PS were expressed as a percentage of the dose recovered in the 0 - 8 hour urine. In this way, the large interindividual variation in the total percentage of dose excreted is removed, and so any intraindividual relationship between PG and PS can be observed. A strong negative correlation ($p < 0.01$) was found between PG and PS excretion when they were expressed as a percentage of the dose recovered. This strongly suggests that the 2 pathways, glucuron

idation and sulphation are in competition with each other during the metabolism of paracetamol. Oldham (1983) reported similar findings.

Effect of age

In the present study no significant correlation was found between age and the 0-8 hour urinary excretion of P, PG and PS. Similarly, Mucklow *et al.* (1980) failed to find a correlation between age and paracetamol plasma clearance and paracetamol half-life. In both studies, the subjects were aged from 18 to 60 years. However, Briant *et al.* (1975), Rawlins *et al.* (1979) and Triggs *et al.* (1975), have all shown that subjects over 65 years old have significantly longer paracetamol half-lives when compared with subjects under 40 years old, indicating that the elderly subjects may have impaired paracetamol metabolism. Triggs *et al.* also studied the urinary excretion of paracetamol and its metabolites and although the percentage of dose excreted in 24 hours was similar in both young and elderly, the percentage of conjugated paracetamol excreted was much more variable in the elderly. Therefore, they suggested that conjugation may be impaired in some elderly subjects. The data presented here, together with that of Mucklow *et al.*, indicates that the capacity for paracetamol conjugation is not affected by age in subjects 18 to 62 years old. However, the data presented by Briant *et al.*, Rawlins *et al.* and Triggs *et al.* suggests that the capacity for paracetamol conjugation is decreased in subjects older than 65 years. Therefore, the lack of effect of age on paracetamol metabolism,

found in the present study, is probably due to the relatively young age range studied. For the effect of age on paracetamol metabolism to be noticed, subjects aged over 65 years should be included in the study.

Effect of body weight

The present study showed that no correlation was found between body weight and 8 - hour urinary excretion of P, PG and PS. Therefore, body weight was found to have no effect upon paracetamol metabolism. This is supported by Mucklow *et al.* (1980), who also failed to find correlations between body weight and paracetamol plasma clearance and paracetamol half-life.

Effect of gender

Males and females were found to excrete similar amounts of paracetamol and its major metabolites, PG and PS, in the present study. Similarly, Miners *et al.* (1980) and Mucklow *et al.* (1980) both found paracetamol plasma clearance to be significantly higher in males than females, although the half-life of paracetamol was found to be similar in both sexes. Furthermore, Miners *et al.* found that the renal and metabolic clearance of PG was significantly higher in males. Therefore, Miners *et al.* stated that the increased paracetamol clearance found in males is due to increased glucuronidation.

However, despite the increased paracetamol clearance found in males, Miners *et al.* found that this sex difference is not confirmed in the total urinary excretion of the paracetamol

metabolites and similar results to those of the present study were obtained. As paracetamol half-life and total urinary excretion of paracetamol and its major metabolites, PG and PS have been found not to differ between males and females, the sex differences found by other workers are probably of little clinical significance.

Effect of smoking

Smoking was found to have no significant effect upon the urinary excretion of PS and P in both males and females. However, although smoking did not affect PG excretion in males, PG excretion was found to be significantly higher in female smokers than in female non-smokers. Therefore, smoking was found to increase paracetamol glucuronidation in the female but had no effect upon paracetamol metabolism in the male.

The inductive effect of smoking on paracetamol metabolism found in the present study is supported by Mucklow *et al.* (1980) who found plasma paracetamol clearance to be significantly higher in smokers than non-smokers.

Cigarette smoke contains polycyclic hydrocarbons which are known to be potent enzyme inducers. Polycyclic hydrocarbons have been found to increase the activity of several microsomal enzyme systems involved in Phase I metabolism, including N-demethylation, hydroxylation and reduction (Conney and Burns, 1962). Hamada and Gessner, 1975, have shown that the polycyclic hydrocarbon, 3-methylcholanthrene increases UDP-glucuronyltransferase activity in rats, whilst Uotila and Marrierni, 1976, have demonstrated increased glucuronidation in the rat lung following inhalation of cigarette smoke for

1 hour.

These studies indicate that, in rats, cigarette smoke is capable of inducing a wide variety of microsomal enzymes, including UDP-glucuronyltransferase. Therefore, the increased paracetamol plasma clearance found in smokers (Mucklow *et al.*, 1980) is probably due to the induction of the major metabolic pathway, namely glucuronidation. This is supported by the present study where female smokers were found to excrete increased amounts of the major metabolite, PG, indicating induced glucuronidation in these subjects.

Effect of oral contraceptive steroids

The present study indicated that urinary excretion of PG tended to be higher in OCS users, but possibly due to the large interindividual variation, this failed to reach significance. Urinary excretion of PS was found to be significantly reduced in OCS users, whilst urinary excretion of free paracetamol was similar in OCS users and control females. As the present study has shown glucuronidation and sulphation of paracetamol to be competing pathways, the significant decrease in PS excretion found in OCS users has to be accompanied by an increase in PG excretion in these women. Hence, in the present study, OCS have been found to alter the metabolism of paracetamol whereby the urinary excretion of PG was increased and PS excretion was decreased. The question arises as to whether the OCS inhibit sulphation resulting in increased glucuronidation or whether they induce glucuronidation resulting in decreased sulphation.

The results of the present study are supported by Abernethy *et al.* (1982); Miners *et al.* (1983); Mitchell *et al.* (1983) and Mucklow *et al.* (1980). They found that OCS cause increased plasma clearance and a shorter half-life of paracetamol. Furthermore Miners *et al.* and Mitchell *et al.* found that OCS caused an increase in the metabolic clearance of PG, but had no effect upon the metabolic clearance of PS and free P. Therefore they concluded that the increased paracetamol metabolism found in OCS users is due to induction of the glucuronidation pathway.

Therefore the increased PG excretion and decreased PS excretion found in OCS users in the present study may be indicative of induction of paracetamol glucuronidation as opposed to inhibition of paracetamol sulphation.

b) Genetic control of paracetamol metabolism

The present study showed considerable interindividual variation in the 0 - 8 hour urinary excretion of unchanged P and its 2 major metabolites, PG and PS. A 4-fold variation in PG excretion was obtained (with 19.5 to 71.5% of the dose excreted as PG). PS excretion varied 8-fold (7.1 to 56.0% of the dose excreted as PS) and unchanged P excretion varied 16-fold (0.4 to 6.0% of the dose excreted as P). Similar variations were also found by Caldwell *et al.* (1980) where the urinary excretion of P, PG and PS was studied in 34 healthy young adults.

Genetic control of glucuronidation

Normal distributions were found for both total PG excretion and the metabolic ratio, P/PG. Therefore the glucuronidation capacity of the population studied is probably not subject to monogenic control but is rather controlled and influenced by a number of genetic and environmental factors.

However, glucuronidation of a number of substrates does appear to be under monogenic control, as seen when individuals with Gilbert's syndrome are studied. Gilbert's syndrome is an inherited disorder, present in about 5% of the population. It is characterised by assymptomatic unconjugated hyperbilirubinaemia. The elevated bilirubin levels found in Gilbert's syndrome are mainly due to decreased hepatic uptake of bilirubin from plasma, and more importantly, decreased UDP-glucuronyltransferase activity (Macklon *et al.*, 1979). As Gilbert's syndrome is an autosomal dominant trait, glucuronidation of bilirubin and certain other substrates is subject to monogenic control.

The metabolism of several compounds which undergo glucuronidation, have been found to differ in subjects with Gilbert's syndrome compared to those who do not have the syndrome, including menthol (Beck and Kiani, 1960) and estradiol benzoate (Aldercreutz and Tikkanen, 1973). Conversely, the plasma half-life and clearance of oxazepam and lorazepam were found to be unaltered in subjects with Gilbert's syndrome even though these 2 benzodiazepines also undergo glucuronidation.

Paracetamol metabolism has also been studied in subjects with Gilbert's syndrome (Douglas *et al.*, 1978). Paracetamol plasma

clearance and the volume of distribution were found to be lowered in these subjects, although the half-life was not altered. Therefore, as the paracetamol half-life is unaffected by Gilbert's syndrome, the question arises as to whether the urinary excretion of PG, one of the major metabolites of paracetamol, would be expected to be lowered.

The present study showed a continuous distribution for paracetamol glucuronidation (when expressed as either the total urinary excretion of PG or as the metabolic ratio P/PG) with no isolated sub-group of "poor glucuronidisers". This could be due to the population studied not containing any subjects with Gilbert's syndrome (although from a population of 99 there would be an expected number of 5). Or, Gilbert's syndrome has no effect upon paracetamol glucuronidation and different forms of UDP-glucuronyl-transferase are involved in bilirubin and paracetamol metabolism. As at least 2 forms of UDP-glucuronyltransferase has been isolated in the rat liver (Burchell, 1977), the latter explanation for the unimodal distribution obtained in the present study, seems the most probable. Studies performed in man also indicate that several isozymes of UDP-glucuronyltransferase may exist. For example, oxazepam and temazepam have both shown sex differences, whereas lorazepam does not, even though all three are benzodiazepines and undergo glucuronidation. Similarly, the metabolism of estradiol and menthol were altered in Gilbert's syndrome but the metabolism of oxazepam and lorazepam were not, even though they too all undergo glucuronidation. The difference in response to gender and Gilbert's syndrome, depending upon the substrate indicates the multiplicity

of the glucuronidase enzyme(s). Therefore, the absence of an isolated sub-group of "poor glucuronidisers" in the population studied, is probably due to different forms of UDP-glucuronyl-transferase being involved in the metabolism of bilirubin and paracetamol.

Genetic control of sulphation

Total PS excretion and the metabolic ratio P/PS were both found to have continuous, unimodal distributions. This indicates that sulphation is subject to multifactorial and polygenic control. Although the distribution of PS excretion was found to be slightly skewed, this deviation from a normal distribution was not supported by the metabolic ratio P/PS and so is of little importance. Therefore, sulphation is probably not subject to monogenic control, but it is influenced by a number of genetic and environmental factors.

The metabolic ratio PG/PS showed a strongly skewed distribution. This indicates that the ratio is subject to polygenic and multifactorial control with one or more factor(s) having a predominant influence. As the distribution is positively skewed, either paracetamol glucuronidation is increased and/or paracetamol sulphation is decreased in a percentage of the population.
The present study has shown glucuronidation and sulphation of paracetamol to be competing pathways. Therefore, the metabolic ratio, PG/PS is a sensitive method for assessing genetic and environmental control of paracetamol glucuronidation and sulphation as an increase in one metabolite will generally result

in a decrease in the other.

The present study has shown that paracetamol metabolism is affected by smoking and oral contraceptives, both of which have been found to increase paracetamol glucuronidation. Hence, both smoking and OCS influence the metabolic ratio, PG/PS and these environmental factors could be partly responsible for the deviation from a normal distribution.

The skewed distribution for the ratio PG/PS could also indicate that there may be monogenic control of the conjugation pathways, but the strong influence of environmental factors on paracetamol metabolism may prevent a polymodal distribution from being seen. That is, the interaction of genetic and environmental factors results in the distribution of the ratio being skewed.

There is some evidence indicating that paracetamol metabolism may be under genetic control. Mucklow *et al.* (1980) found that paracetamol clearance and half-life were decreased in Asians compared to Caucasians. However, they stated that the major cause of this apparent racial difference was due to contrasting use of social drugs, including alcohol, tobacco and the contraceptive pill. However, Sommers *et al.* (1985) studied paracetamol metabolism in African villagers and found that paracetamol clearance was significantly increased when compared to the Asians studied by Mucklow *et al.* As both the Africans and Asians had minimal use of social drugs and both were vegetarians, environmental factors cannot explain the racial difference found. Therefore, it would appear that this ethnic variation is indicative of genetic control of paracetamol metabolism. Further indications of genetic control

were found by Caldwell *et al.* (1980) and Shively and Vesell (1975) who found that paracetamol metabolism was highly reproducible in subjects studied on 2 separate occasions.

Therefore, glucuronidation and sulphation of paracetamol may be subject to genetic control although the present study provides no supporting evidence. The glucuronidation and sulphation ratios, P/PG and P/PS, are both normally distributed indicating polygenic and multifactorial control. It is only the more sensitive ratio, PG/PS, which shows a skewed distribution, indicating the predominant control of one or more factors. Environmental factors have been found to be at least partly responsible for this deviation from a normal distribution, and if any genetic factors do contribute to this deviation, their influence is not great enough to be of any clinical importance.

CHAPTER FIVE

THE EFFECT OF CHRONIC ORAL CONTRACEPTIVE USE ON ENDOGENOUS ESTRADIOL METABOLISM

5.1 INTRODUCTION

Approximately 60 million women world-wide use oral contraceptive steroids. The most common formulations are the combined oral contraceptive agents which contain an estrogen and a progestogen. Prolonged use of the combined oral contraceptive pill has been found to alter the metabolism of a wide variety of exogenously administered compounds including antipyrine (Abernethy and Greenblatt, 1981), diazepam (Abernethy *et al.*, 1982) and paracetamol (Miners *et al.*, 1983). However, there have been few reports on the effects of oral contraceptive steroids (OCS) on the metabolism of the endogenous steroid estradiol, and these include Longscope *et al.* (1974) and Femino *et al.* (1974), studying the effect of OCS use on estradiol clearance in females and Slikker *et al.* (1983) studying the effect of OCS use on the clearance and metabolism of estradiol in monkeys.

The endogenous hormone estradiol is the most active natural estrogen and is also very similar in structure to ethinyl estradiol, EE_2, the most common estrogenic component of the OCS. In order to study the effects of chronic OCS use on endogenous metabolism, estradiol was the endogenous compound of choice. Estradiol undergoes numerous metabolic transformations (see Figure 1.4) with the majority undergoing phase I transformation into the

less active compounds, estrone and estriol, both of which are further inactivated by phase II metabolism. A small percentage of estradiol (approx. 2 - 4%) is subject to direct inactivation by phase II metabolism (Beer and Gallagher, 1960; Femino *et al.*, 1974) and it is the direct sulphate and glucuronide conjugation of estradiol which was examined in the present study. In this way, the effect of chronic OCS use on the phase II metabolism of an endogenous compound, the hormone estradiol, was studied.

5.2 PROCEDURE

37 women who had used oral contraceptive steroids for a minimum of 6 months (OCS users) and 8 women who had never used oral contraception (control females) participated in the study. A single 10 ml blood sample and a 24-hour urine were obtained during the later phase of the menstrual cycle. For the OCS users, samples were taken from day 18 to day 21 (i.e. at the end of the pill-taking cycle) whilst for control females samples were taken from day 19 to day 22 (i.e. around the estradiol peak of the luteal phase). Plasma estradiol together with urinary free estradiol (E_2), estradiol glucuronide (E_2G) and estradiol sulphate (E_2S) were assayed by RIA as described in Chapter 2.

The mean age of the OCS users was 21.4 ± 0.5 years (with a range of 18 - 32 years) and their mean weight was 59.7 ± 1.1 kg (with a range of 48 - 75 kg). Similar values were found with the control females. They had a mean age of 22.75 ± 1.5 years (with a range of

21 - 32 years) and a mean weight of 61.75 ± 2.9 kg (with a range of 53 - 72 kg). Individual details of the females who participated in this study, concerning age, body weight, concomitant drug treatment, length and type of OCS use are given in Appendix 3. The Mann-Whitney test was used throughout this study as the test for significance.

5.3 RESULTS

The plasma estradiol concentrations of both control females and OCS users are given in Table 5.1 and Figure 5.1. The plasma concentration found in the OCS users was significantly lower than those of the control females ($p < 0.001$).

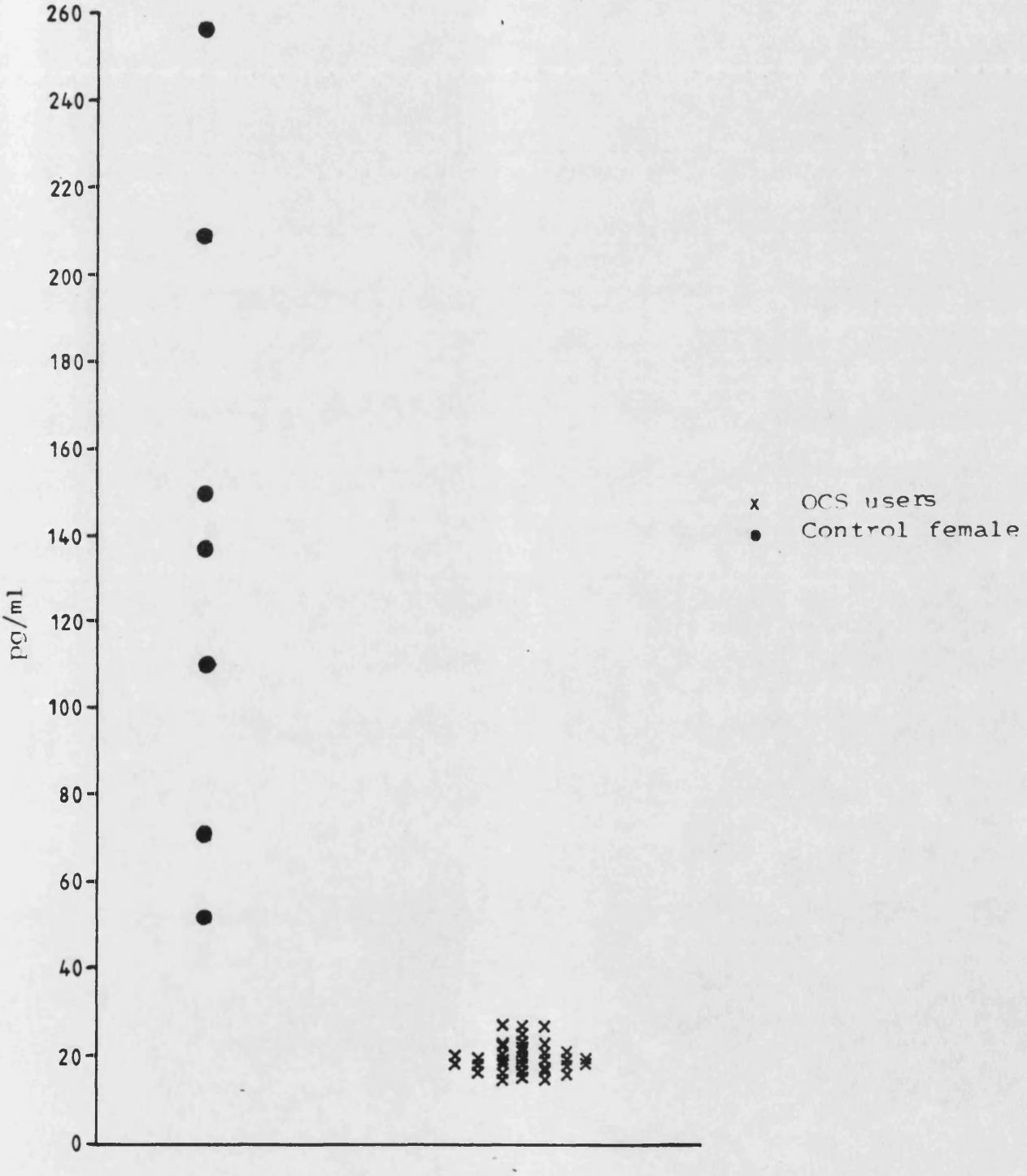
Table 5.1. Plasma estradiol (pg/ml) in control females and OCS users.

	Mean ± SE	Range
Control females (n = 8)	139.9 ± 27.6	51.1 - 256.2
OCS users (n = 37)	18.7 ± 0.5*	14.3 - 26.5

* $p < 0.001$ when compared to control

FIGURE 5.1

The Scattergram for serum estradiol in females.



The 24-hour urinary excretion of total estradiol and its individual metabolites, free estradiol (E_2), estradiol glucuronide (E_2G) and estradiol sulphate (E_2S) for both control females and OCS users is given in Table 5.2

Table 5.2. The 24-hour urinary excretion of estradiol and its direct conjugates E_2G and E_2S in control females and OCS users (n = 8 and 37 respectively).

	Mean ± SE (μg)		Range (μg)	
	CONTROL	OCS USER	CONTROL	OCS USER
Total E_2	4.67 ± 0.97	0.93 ± 0.24**	1.78 - 10.47	0.22 - 6.99
Free E_2	0.59 ± 0.35	0.14 ± 0.01*	0.09 - 2.99	0.06 - 0.30
E_2G	3.01 ± 0.49	0.53 ± 0.10**	1.24 - 5.49	0.09 - 2.75
E_2S	1.16 ± 0.23	0.29 ± 0.15**	0.44 - 1.99	0.00 - 4.43

* $p < 0.05$ when compared to control

** $p < 0.01$ when compared to control

The excretion of total estradiol, E_2G and E_2S were all found to be significantly lower in OCS users than in control females ($p < 0.001$). The excretion of free estradiol was also lower in OCS users than in the control females ($p < 0.05$).

The relationship between the urinary excretion of free E_2 and its 2 direct phase II metabolites, E_2G and E_2S in both control females and OCS users are shown in Figs. 5.2 and 5.3. Due to the large

FIGURE 5.2

Relationship between the urinary excretion of free E_2 and E_2G in OCS users and control females.

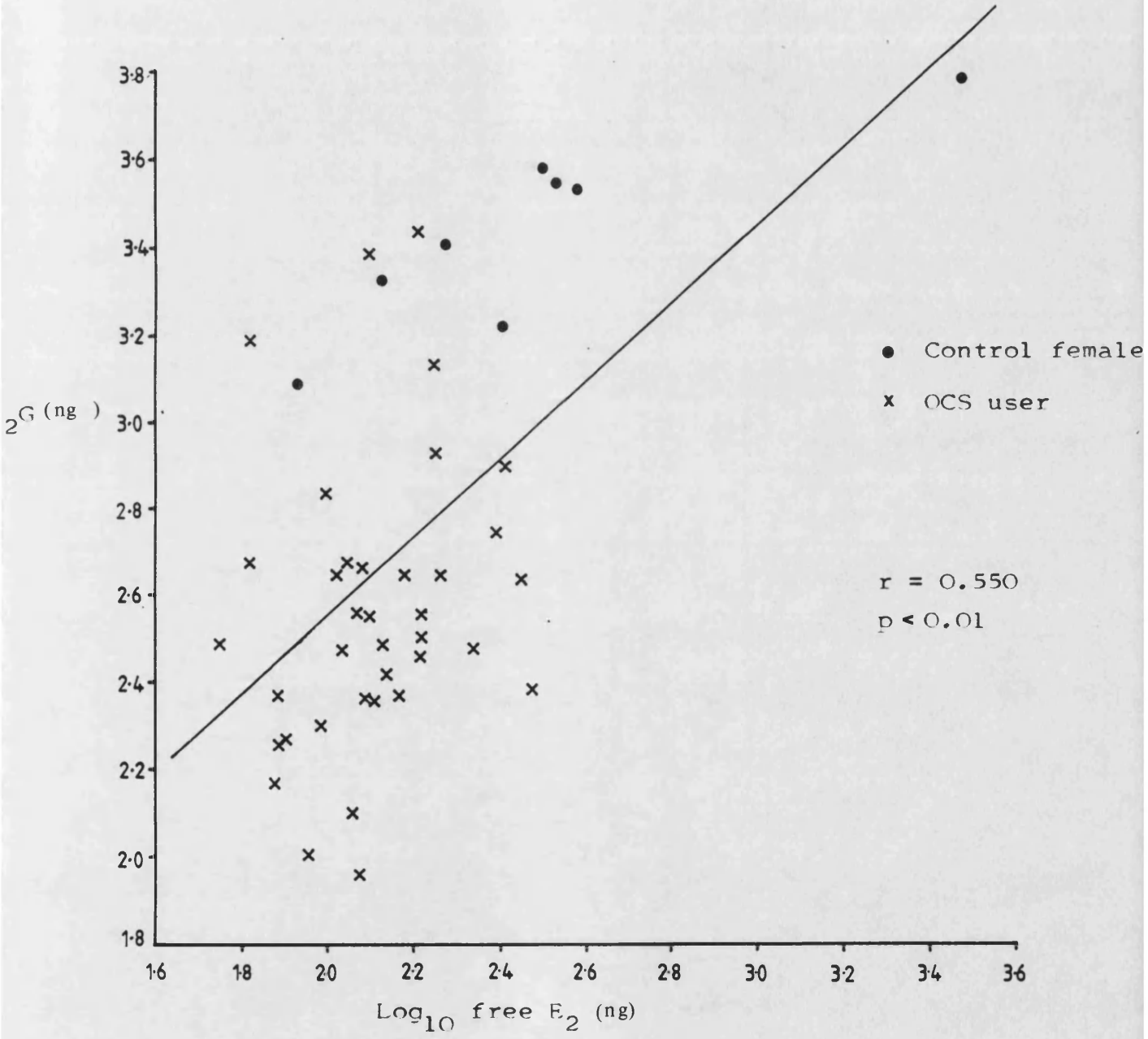
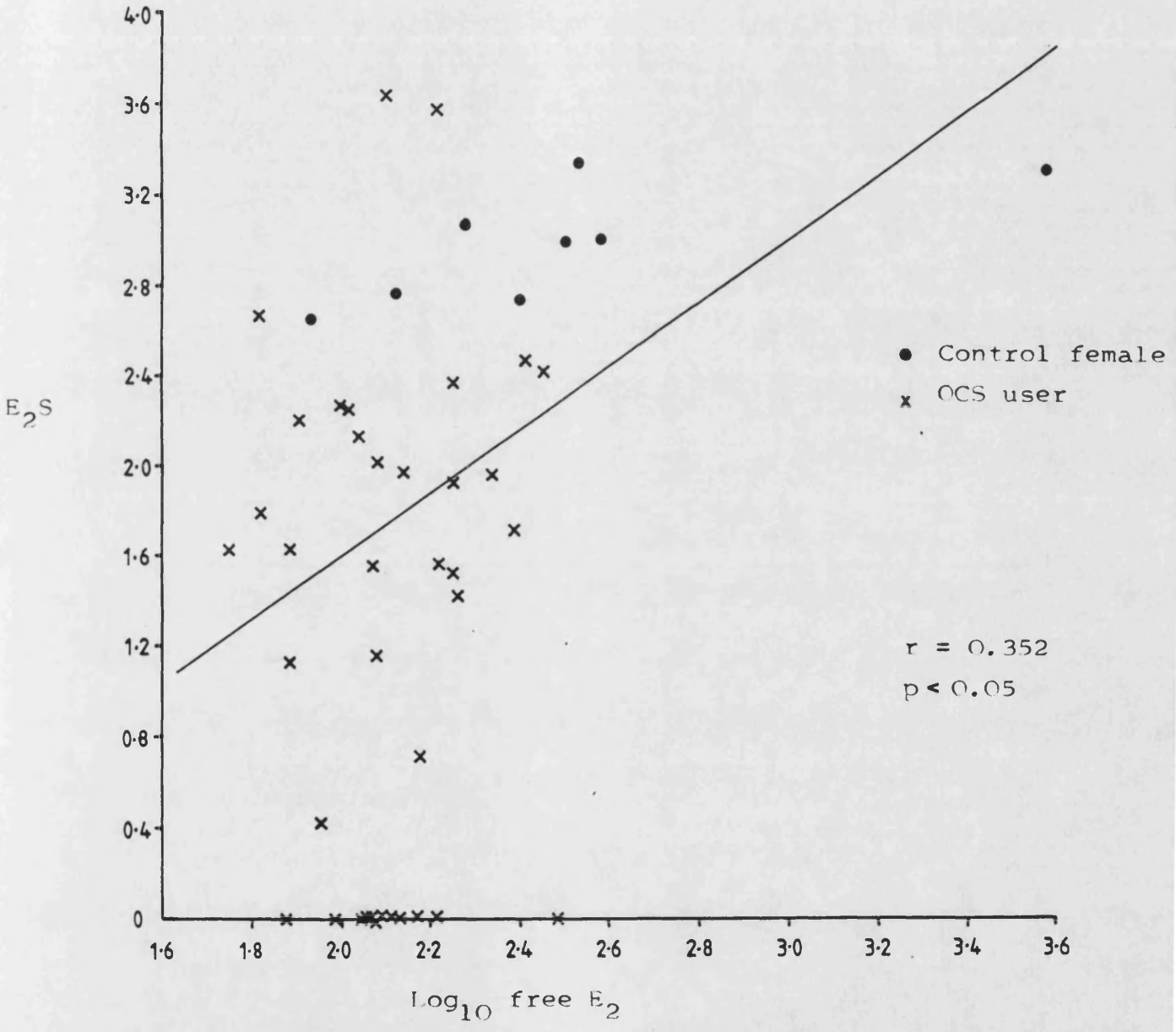


FIGURE 5.3

Relationship between the urinary excretion of free E_2 and E_2S in OCS users and control females.



variation in excretion of these 3 compounds, the scattergrams are expressed on a $\log_{10}$ scale. A weak positive correlation was found between the $\log_{10}$ urinary excretion of free E_2 and E_2S ($r = 0.352$, $p < 0.05$) but a stronger correlation was found between the $\log_{10}$ urinary excretion of free E_2 and E_2G ($r = 0.550$, $p < 0.001$). Further study of these scattergrams show that only 50% of the control females excreted elevated amounts of free E_2 when compared to OCS users, indicating considerable overlap in free E_2 excretion between the 2 groups. In contrast, 89% of OCS users excreted lower amounts of E_2G and E_2S when compared to control females, indicating little overlap in E_2G and E_2S excretion between the 2 groups. Fig. 5.4 shows the relationship between the urinary excretion of E_2G and E_2S in both groups and it indicates that there is a positive correlation ($r = 0.8011$, $p < 0.001$).

The metabolic ratios, free E_2/E_2G, free E_2/E_2S and E_2G/E_2S give an indication of the extent to which estradiol undergoes glucuronidation and sulphation and these ratios for both control females and OCS users are given in Table 5.3.

The ratio E_2/E_2G was found to be significantly higher in the 37 OCS users when compared to controls ($p < 0.05$). However, as some of the subjects who use OCS were found to excrete no estradiol sulphate, they had to be omitted when calculating E_2/E_2S and E_2G/E_2S since the ratios would be ∞.

FIGURE 5.4

Relationship between the urinary excretion of E_2G and E_2S in OCS users and control females.

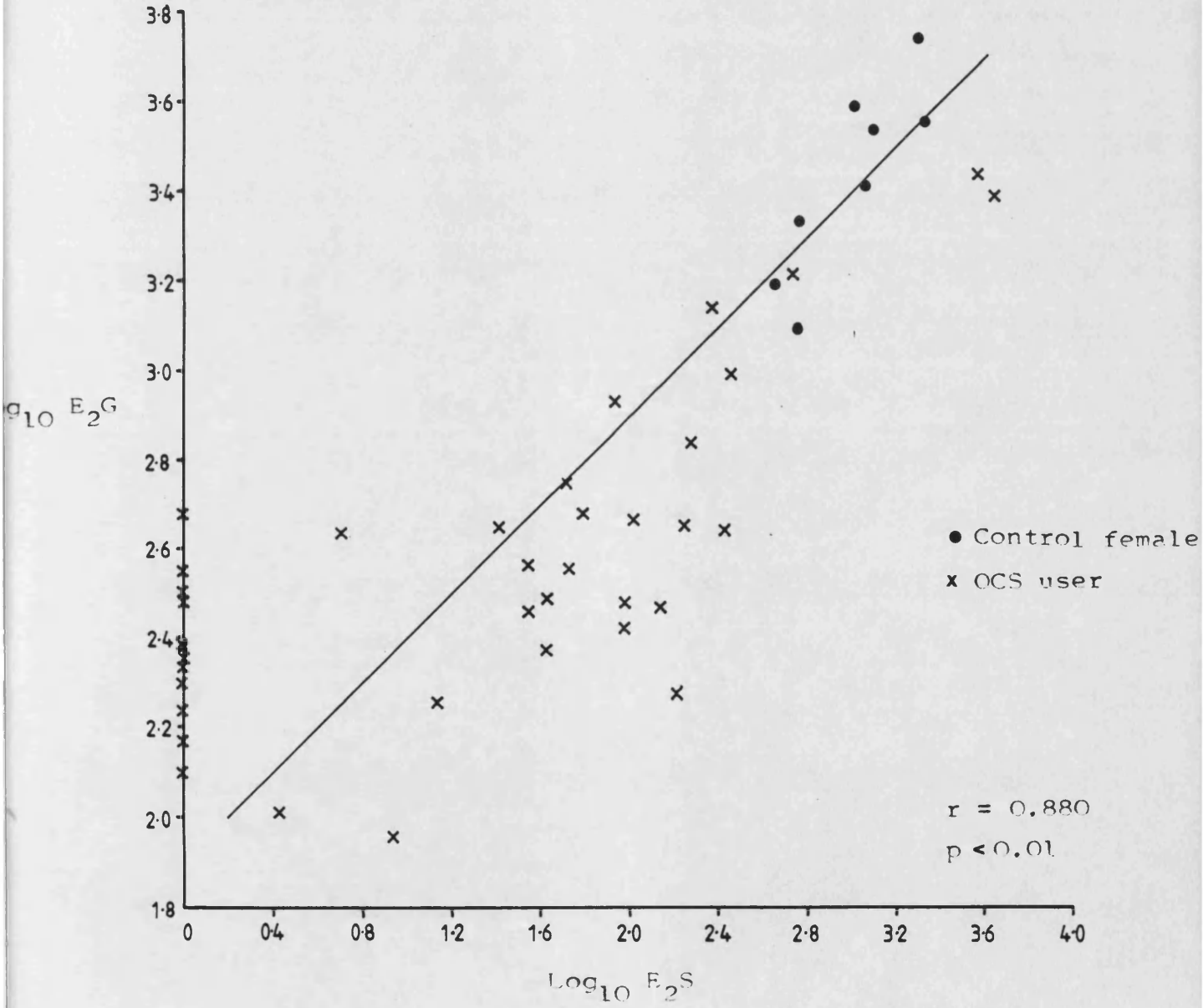


Table 5.3: The ratios E_2/E_2G, E_2/E_2S and E_2G/E_2S in control females and OCS users.

	Mean ± SE		Range	
	CONTROL	OCS USER	CONTROL	OCS USER
E_2/E_2G	0.148±0.058	0.449±0.049*	0.062-0.546	0.052-1.229
E_2/E_2S	0.411±0.16	3.38±1.205*	0.115-1.507	0.029-34.00
E_2G/E_2S	2.833±0.258	10.331±3.38*	2.160-3.89	0.55 - 37.70

* $p < 0.05$

Therefore, the metabolic ratios E_2/E_2S and E_2G/E_2S were found to be significantly higher in the 26 OCS users (who had E_2S values > 0) than the control females ($p < 0.05$). Figs. 5.5a to c show the scattergrams for the metabolic ratios E_2/E_2G, E_2/E_2S and E_2G/E_2S and the spread for each of the ratios appears to be much greater for the OCS users than the control females. There is little overlap between the 2 groups for the metabolic ratios E_2/E_2G and E_2/E_2S with only 11% of the OCS users having ratios less than those found for the majority of the control females. However, the overlap appears to be greater for the metabolic ratio E_2G/E_2S with 30% of the OCS users having ratios equal or less than those of the control females.

FIGURE 5.5a

The Scattergram for the metabolic ratio, $\frac{E_2}{E_2G}$ in human urine.

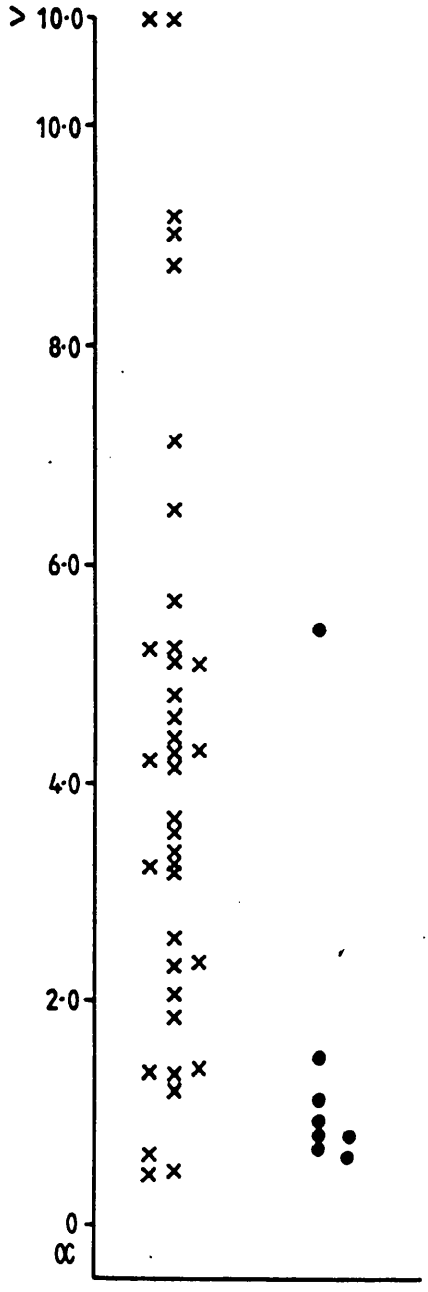


FIGURE 5.5b

The Scattergram for the metabolic ratio, $\frac{E_2}{E_2S}$ in human urine.

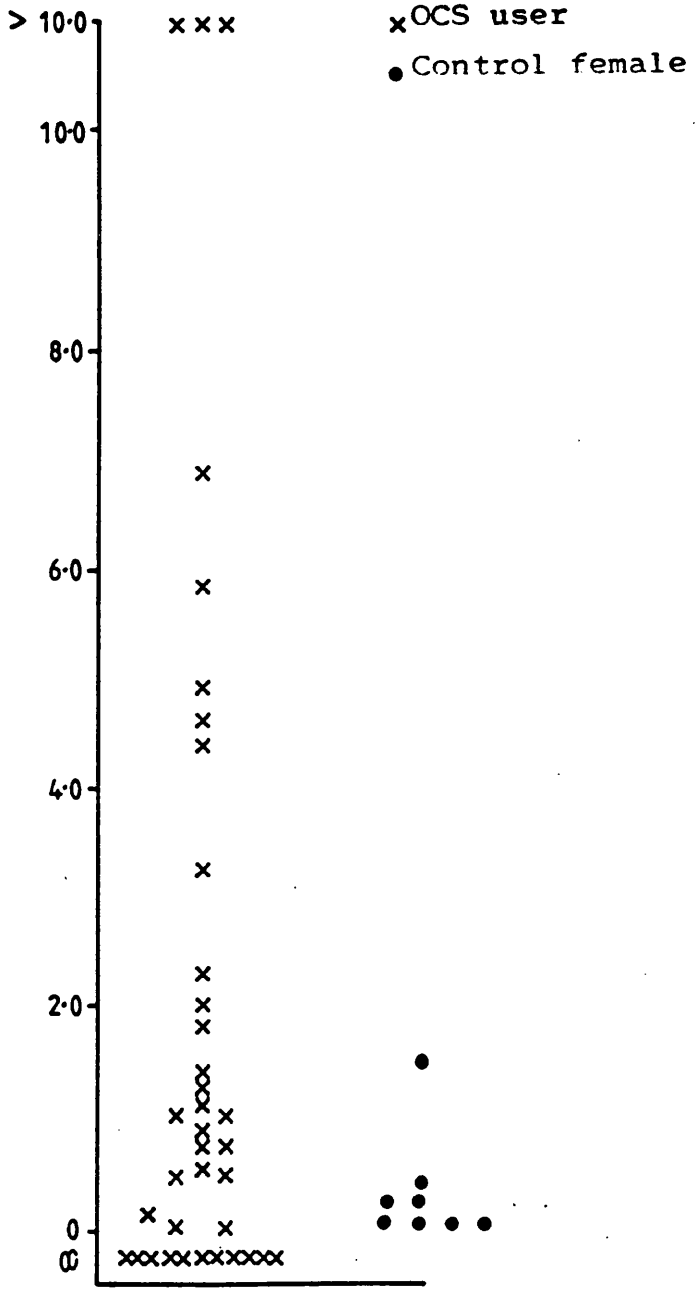
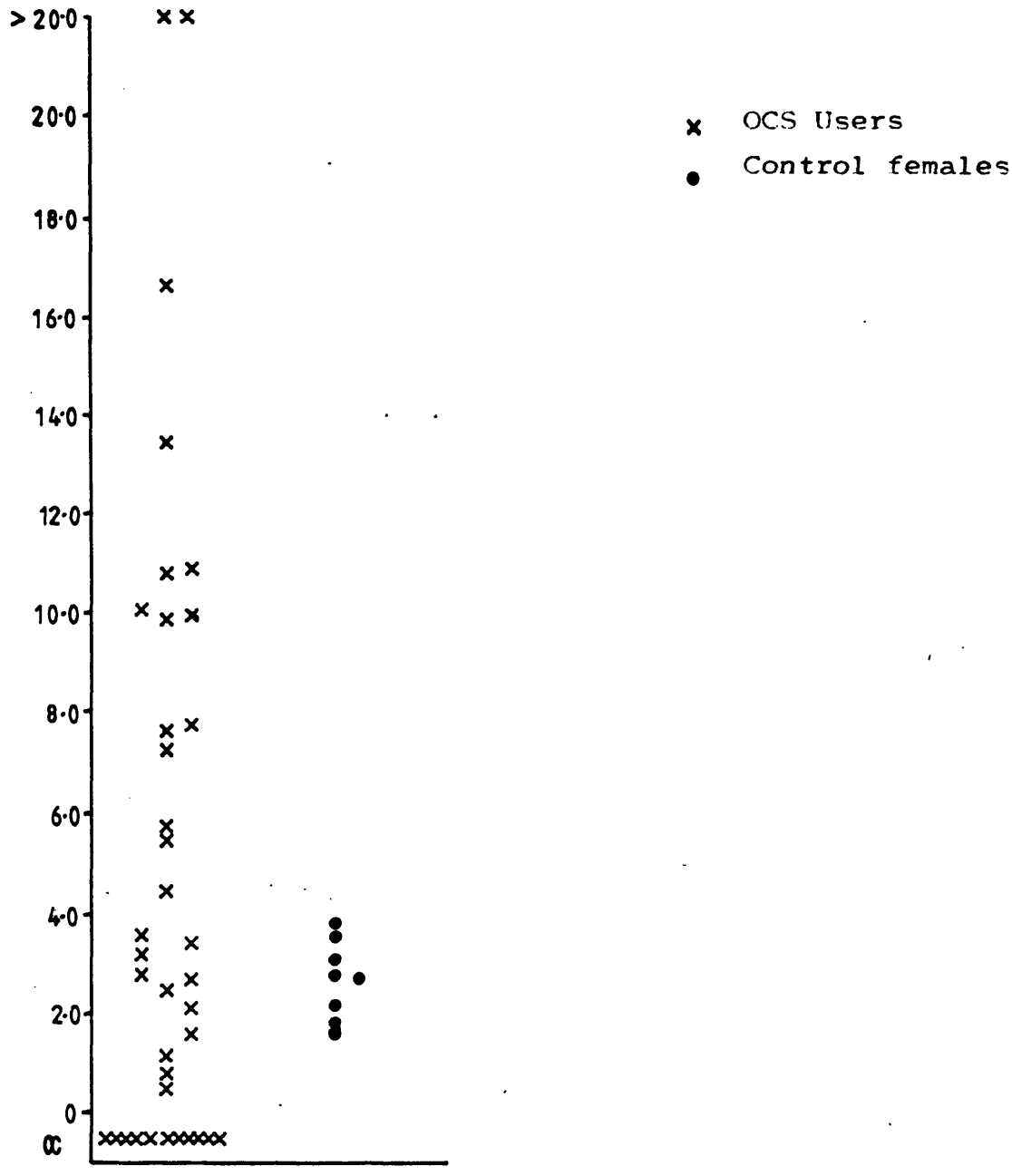


FIGURE 5.5c

The Scattergram for the metabolic ratio, $\frac{E_2G}{E_2S}$ in human urine.



It is of interest to note that the control female whose values for E_2, E_2G and E_2S excretion were 5 to 10 fold higher than the other control females had been subject to deep vein thrombosis which required hospital treatment. None of the other control females had any personal or family history of thrombosis. In contrast, the few OCS users who had elevated values for E_2G and E_2S excretion in comparison to the other OCS users had no distinguishing details to account for these differences.

5.4 DISCUSSION

In the present study, the control females were found to have luteal phase serum estradiol levels of 139.9 ± 27.6 pg/ml (mean ± SE), and they excreted 4.67 ± 0.97 µg of total estradiol in 24 hours. Similar values have been found by several workers including Brown (1960); Knorr *et al.* (1970) and Fels *et al.* (1979). The present study has also found that OCS users have significantly lower serum estradiol levels (18.7 ± 0.5 pg/ml; $p < 0.001$) together with a significantly lower 24-hour urinary excretion of total estradiol (0.93 ± 0.24 µg; $p < 0.01$). However, the sampling times for OCS users and the control females are not necessarily equivalent in terms of cycle period, which could partially contribute to the significant differences in the plasma concentration and urinary excretion of estradiol.

The decrease in estradiol concentrations caused by OCS found in the present study is supported by several other workers, and this effect is due to the activity of the drug. Oral contraceptives act

on the hypothalamus and pituitary gland and prevent ovulation by suppressing the mid-cycle increase of LH and FSH levels. However, the OCS are also thought to have a direct action on the ovaries. Mishell *et al.* (1972) and Kjeld *et al.* (1976) have shown that during OCS use the plasma concentration of estradiol is decreased and both the ovulatory peak and the luteal phase rise are uniformly suppressed. Similarly, the urinary excretion of total estrogens and unconjugated estradiol levels have been found to be significantly decreased by OCS use, as reported by Loraine *et al.* (1965) and Kjeld *et al.* (1976) respectively. Furthermore, the estradiol concentrations have been found to remain low during the initial cycle of OCS use, and so OCS are thought to have a direct action on the ovary whereby estradiol production is decreased. The present study has shown that OCS reduce both plasma estradiol and urinary total estradiol levels which is to be expected since OCS suppress the production of estradiol.

OCS are known to affect the metabolism of many exogenous compounds including antipyrine (Abernethy and Greenblatt, 1981), diazepam (Abernethy *et al.*, 1982) and paracetamol (Miners *et al.*, 1983), but little is known about their effect on the metabolism of endogenous compounds. Therefore, the urinary excretion of free estradiol and 2 metabolites, EG and ES was studied. OCS users were found to excrete significantly less free E_2, E_2G and E_2S than the control females ($p < 0.05$, $p < 0.01$, $p < 0.01$ respectively). However, as a significant decrease in estradiol concentrations (both plasma and urinary) was found in OCS users compared to control females, significant decreases in the urinary excretion of each of the

metabolites is to be expected.

The urinary ratios E_2/E_2G and E_2/E_2S provide an indication of the extent of glucuronidation and sulphation of estradiol. The ratio E_2/E_2G was 0.449 ± 0.049 in OCS users and 0.148 ± 0.058 in control females whilst the ratio E_2/E_2S was 3.38 ± 1.205 in OCS users and 0.411 ± 0.160 in control females. Hence both ratios were found to be significantly higher in OCS users compared to the control females ($p < 0.05$ for both). The present study has shown that the urinary excretion of all 3 metabolites, free E_2, E_2G and E_2S is significantly lowered by OCS use. Therefore, the significant increases in the ratios E_2/E_2G and E_2/E_2S indicates that the excretion of the 3 metabolites are not decreased to the same extent by OCS use. The increases in the ratios may be due to:

Either: the decrease in free E_2 excretion being comparatively less than the decreases in E_2G and E_2S excretion;

Or: the decreases in E_2G and E_2S excretion being comparatively greater than the decrease in free E_2 excretion.

Figures 5.2 and 5.3 indicate that there is little overlap between OCS users and control females in the urinary excretion of both E_2G and E_2S, with 87% of OCS users excreting less E_2G and E_2S than the control females. In contrast, these same figures show considerable overlap between the 2 female groups in the urinary excretion of free estradiol, despite the significant decrease in free E_2 excretion found in OCS users. Therefore the large overlap found in free E_2 excretion but not in E_2G and E_2S excretion may indicate that the decrease in free E_2 excretion is comparatively less than the decrease in E_2G and E_2S excretion, which may account for the

significant increases in the ratios E_2/E_2G and E_2/E_2S found in OCS users.

Although a small percentage of estradiol undergoes glucuronide and sulphate conjugation, the majority undergoes extensive oxidative biotransformation. The oxidative products of estradiol have not been studied in the present work; nevertheless, inhibition of one or more of the oxidative pathways may result in OCS causing a relatively smaller decrease in free E_2 excretion compared to the decreases in E_2G and E_2S excretion. This could, in turn, account for the significant increases in the ratios E_2/E_2G and E_2/E_2S found in the present study. The proposal that inhibition of the oxidative pathways of estradiol may be ultimately responsible for the increase in the metabolic ratios, has support from several workers who have examined the effects of OCS on the phase I metabolism of numerous exogenous and endogenous compounds. Chronic OCS use has been found to impair the metabolic clearance and prolong the elimination half-life of many drugs which undergo oxidation. These include antipyrine (Abernethy and Greenblatt, 1981); diazepam (Abernethy *et al.*, 1982); chlordiazepoxide (Roberts *et al.*, 1979), nitrazepam (Jockemsen *et al.*, 1982); caffeine (Patwardhan *et al.*, 1981) and theophylline (Tomatore *et al.*, 1982).

Furthermore, the effect of chronic OCS use on the metabolism of exogenously administered estradiol has been studied (Femino *et al.*, 1974). A decrease in the urinary excretion of the 16 hydroxylated metabolites (namely 16 hydroxyestrone and estriol) was found in OCS users together with a decrease in the total urinary recovery of the administered estradiol.

These studies have shown that combined OCS impair hepatic microsomal oxidation of both exogenous and endogenous compounds. This inhibition of phase I metabolism only occurs with OCS containing EE_2; it is not found in mestranol-containing OCS nor in progestogen-only preparations. Therefore, it would appear that EE_2 is responsible for the decrease in oxidation found in OCS users. Furthermore, *in vitro* studies have shown that EE_2 treatment decreases cytochrome P_{450} levels and turnover in rats (Mackinnon *et al.*, 1977).

Hence, the results of these studies indicate that chronic use of OCS can result in impairment of phase I metabolism. This supports the proposal that the increase in the metabolic ratios E_2/E_2G and E_2/E_2S found in OCS users may be ultimately due to a decrease in the oxidation of estradiol. The oral contraceptives may inhibit some oxidation pathways of estradiol (with Femino *et al.* (1974) indicating that 16 hydroxylation is decreased by chronic OCS use) resulting in the decrease in free E_2 excretion (as caused by OCS suppressing estradiol production) being comparatively smaller than the decrease in E_2G and E_2S excretion, which in turn results in increased ratios, E_2/E_2G and E_2/E_2S.

The metabolic ratio E_2G/E_2S was found to be significantly increased by OCS use in the present study ($p < 0.05$), which indicates that the oral contraceptives cause either an increase in the glucuronidation of estradiol or a decrease in the sulphation. However, OCS have been found to produce significant decreases in the urinary

excretion of both E_2G and E_2S; although this effect is known to be due to the contraceptive activity of the steroids whereby estradiol synthesis is maintained at a low level. Therefore, the significant increase in the ratio E_2G/E_2S has to be due to either a relative increase in estradiol glucuronidation in comparison to sulphation, or a relative decrease in estradiol sulphation in comparison to glucuronidation.

Several workers have studied the effects of OCS use on several phase II conjugation reactions. Miners *et al.* (1983) found that OCS use resulted in induction of paracetamol glucuronidation whilst paracetamol sulphation was unaffected. A similar result was found in the paracetamol population study discussed in Chapter 4. Miners *et al.* (1984), also found that OCS induce glucuronidation of clofibric acid. Patwardhan *et al.* (1981) discovered that the clearance of lorazepam and oxazepam was significantly increased in OCS users and as the major metabolic pathway of these 2 benzodiazepines is glucuronidation, they proposed that glucuronidation was induced during OCS use. Similarly, Ochs *et al.* (1984) found that acetominophen clearance is increased during OCS use and as this compound undergoes glucuronidation and sulphation, one or both pathways have been induced by OCS use.

Hence these studies show that chronic OCS use can result in induction of glucuronidation and to date there is no evidence for OCS causing inhibition of any phase II reactions. Therefore, it would seem likely that the increase found in the metabolic ratio, E_2G/E_2S may be due to induction of estradiol glucuronidation rather than inhibition of estradiol sulphation. Thus as OCS are known to

cause induction in the glucuronidation of several exogenous compounds including paracetamol, clofibric acid, lorazepam, oxazepam and acetominophen, they may also increase the glucuronidation of the endogenous compound, estradiol. The relative increase in E_2G excretion in comparison to E_2S excretion as found in the present study indicates that glucuronide induction may have occurred but further work must be done to confirm this.

Chronic OCS use may result in induction of estradiol glucuronidation, but the metabolic ratio E_2/E_2G was found to be increased in OCS users and not decreased as would be expected if the excretion of E_2G was elevated in these subjects. This discrepancy is probably due to only a small percentage of estradiol undergoing direct conjugation with glucuronide and sulphate. The majority of estradiol undergoes oxidative biotransformation prior to conjugation and subsequent urinary excretion. Consequently, if OCS do cause inhibition of some of the oxidative pathways the resultant increase in free E_2 levels may be greater than the inductive effect of these steroids on the glucuronidation pathway causing a decrease in free E_2 levels, as oxidation is the major metabolic biotransformation of estradiol whilst direct conjugation is relatively minor.

CHAPTER SIX

THE EFFECT OF CHRONIC ETHINYLESTRADIOL PRETREATMENT ON PARACETAMOL AND ESTRADIOL METABOLISM IN THE FEMALE RAT

6.1 INTRODUCTION

Chronic use of oral contraceptive steroids (OCS) has been shown to increase paracetamol clearance, due to induction of both the glucuronidation and oxidation pathways but no change in the sulphation pathway was reported (Miners *et al.*, 1983). Similarly, chronic OCS use has been found to increase the clearance of exogenously administered estradiol (Longscope and Williams, 1974; Slikker *et al.*, 1984), which Slikker *et al.* have indicated is due to induction of the estradiol metabolites, estrone and estrone glucuronide formation. However, the component of the OCS that is responsible for this induction is not known. Therefore, the effect of the estrogenic element, ethinylestradiol (EE_2), on the metabolism of paracetamol and estradiol in the female rat was studied with a view to obtaining the effect of EE_2 on exogenous and endogenous metabolism.

6.2 PROCEDURES

Female Wistar rats weighing 200 to 220 g were used for the studies; n = 24 for the paracetamol study and n = 17 for the estradiol study. All of the rats were treated orally for 40 days; half of the rats receiving 1 ml of 5% ethanol (control rats) and the other half receiving 1 ml of EE_2 solution (11 µg EE_2/ml in 5% ethanol; test

rats).

For the paracetamol study on day 29 of dosing, all 24 rats received a single oral dose of paracetamol (50 mg/kg) and urine was collected from 0 - 8 and 8 - 24 hours. On day 40 a second oral dose of paracetamol (50 mg/kg) was administered and the animals were bled by cardiac puncture over 8 hours at 10, 15, 30, 45, 60, 90, 120, 180, 210, 240, 360 and 480 minutes. Each animal was bled up to 4 times, with a maximum of 2 mls of blood taken on each occasion. Each bleed was followed by a 2 ml subcutaneous injection of 0.9% NaCl. Paracetamol (P), paracetamol glucuronide (PG) and paracetamol sulphate (PS) were analysed in the urine by HPLC together with paracetamol in serum as described in 2.8a and b.

For the estradiol study on day 40, 3 of the control rats and 4 of the test rats were cannulated and ^{14}C-17β-estradiol (26.6 μg E_2; 5.075 μCi/kg) was administered through the jugular vein and the animals were bled from the carotid artery over 3 hours at 1, 2, 4, 6, 8, 10, 13, 16, 20, 30, 45, 60, 90, 120, 150, 180, 210 and 240 minutes. Each animal had up to 7 blood samples taken, with a maximum of 0.5 ml of blood taken on each occasion. The remaining rats had ^{14}C-17β-estradiol injected into the tail vein, also on day 40 and the urine and faeces were collected for 24 hours. At the end of the 24 hour urine collection, the animal was sacrificed and the liver, kidney and the small and large intestine were removed. The radioactivity in the serum, urine, faeces and tissues was determined using the liquid scintillation counter as described in 2.1.

For both studies the Mann-Whitney test was used to assess statistical significance.

6.3 PARACETAMOL STUDY

a) Results

The effects of chronic EE_2 pretreatment on the urinary excretion of paracetamol and its major metabolites PG and PS in the rat are shown in Figs. 6.1 to 6.4, and Table 6.1.

The EE_2-treated rats were found to excrete more PG in 0 to 8 hours than the control rats ($p < 0.05$) with P and PS excretion being similar in both groups. Furthermore, the total dose recovered in the 0 - 8 hour urine was found to be in the EE_2-treated rats but this difference failed to reach significance. In the 8 to 24 hour urine collection, EE_2-treated rats excreted significantly less PS than control rats ($p < 0.05$) but P and PG excretion showed no difference between the 2 groups. The total dose recovered in this time period was significantly in EE_2-treated rats ($p < 0.05$) than in non-treated rats. The EE_2-treated rats were found to excrete more PG and less PS than the control rats in the 0 to 24 hour urine, but these differences failed to reach significance. Total recovery in this time period, together with the free P excretion were similar in both groups.

Table 6.2 and Figs. 6.5a and b show the effects of chronic EE_2 pretreatment on the serum clearance of paracetamol.

Table 6.1. The urinary excretion of P, PG and PS in control and test rats. (Results expressed as % of dose recovered; mean ± SE).

	CONTROL			TEST		
	0-8 hours	8-24 hours	0-24 hours	0-8 hours	8-24 hours	0-24 hours
P	1.8 ± 0.1	1.3 ± 0.3	3.1 ± 0.3	2.0 ± 0.2	1.1 ± 0.3	3.0 ± 0.4
PG	11.1 ± 1.1	2.7 ± 0.5	14.0 ± 1.0	16.0 ± 1.7*	2.8 ± 0.6	18.2 ± 1.8
PS	40.9 ± 3.5	19.1 ± 2.2	60.0 ± 3.0	45.1 ± 3.5	12.3 ± 1.8*	55.6 ± 3.5
P+PG+PS	53.9 ± 4.4	23.2 ± 2.4	77.1 ± 3.6	63.1 ± 5.2	16.3 ± 2.3*	76.9 ± 5.3

* $p < 0.05$ when compared to control.

FIGURE 6.1

The urinary excretion of free paracetamol in female rats.

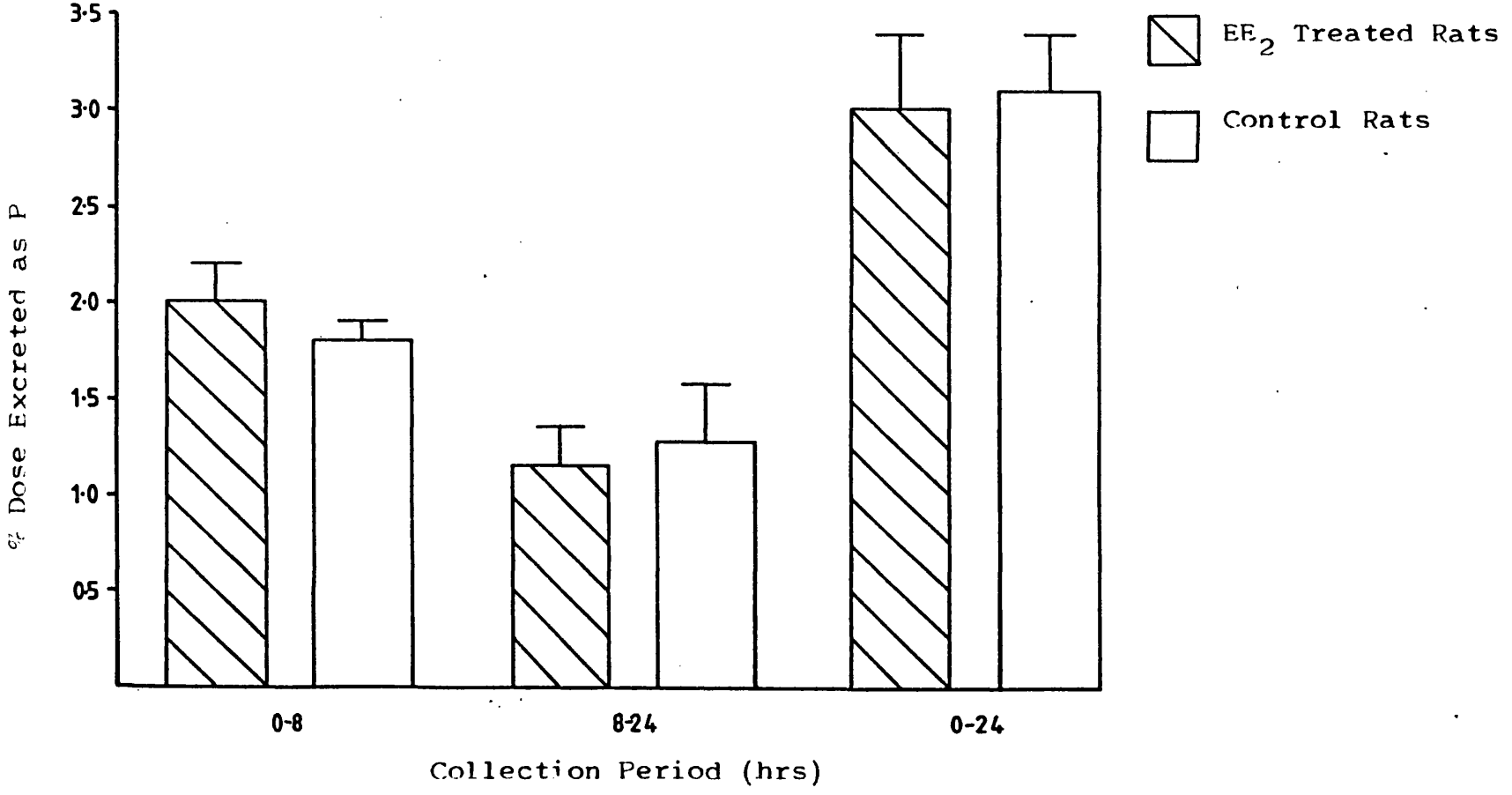


FIGURE 6.2

The urinary excretion of PG in female rats.

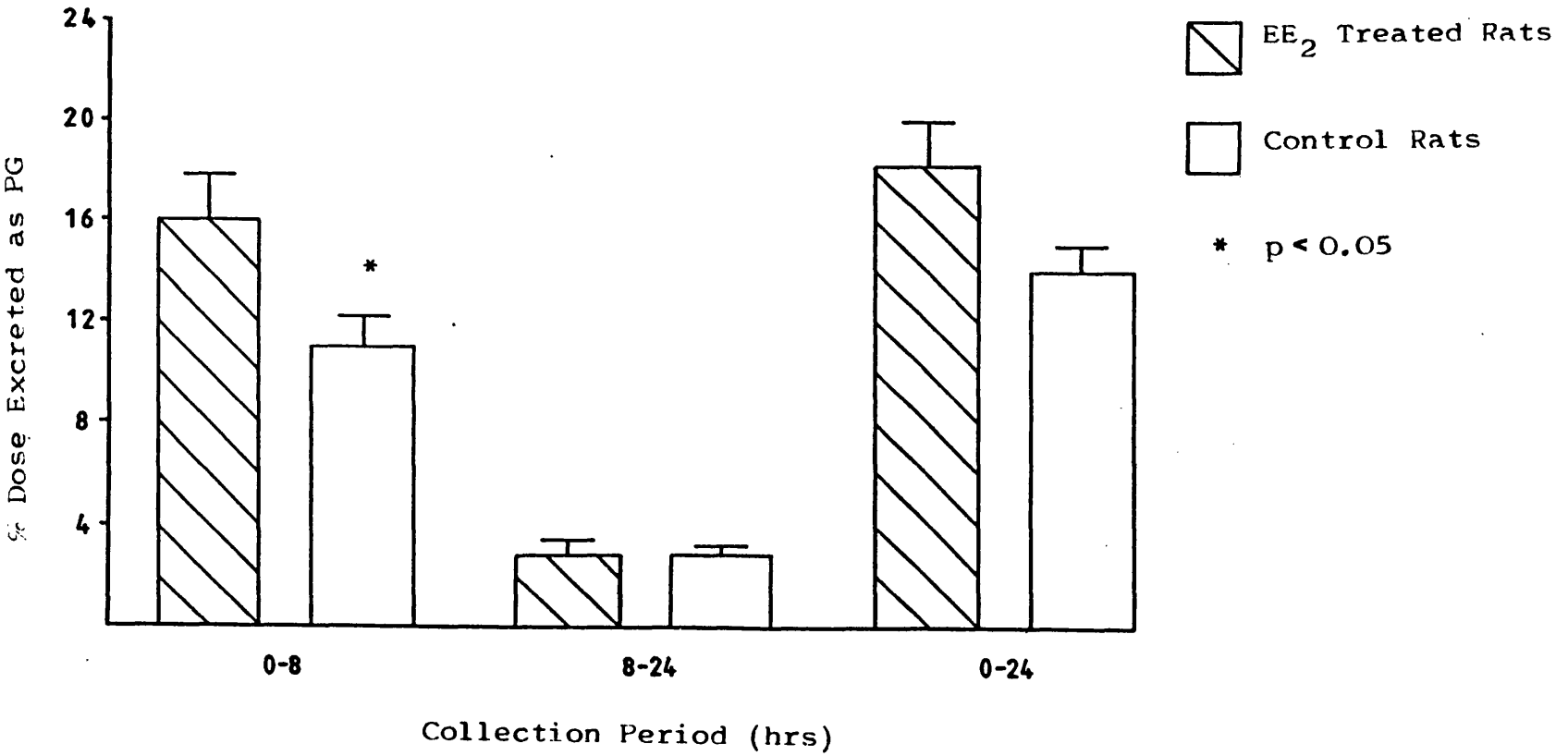


FIGURE 6.3

The urinary excretion of PS in female rats.

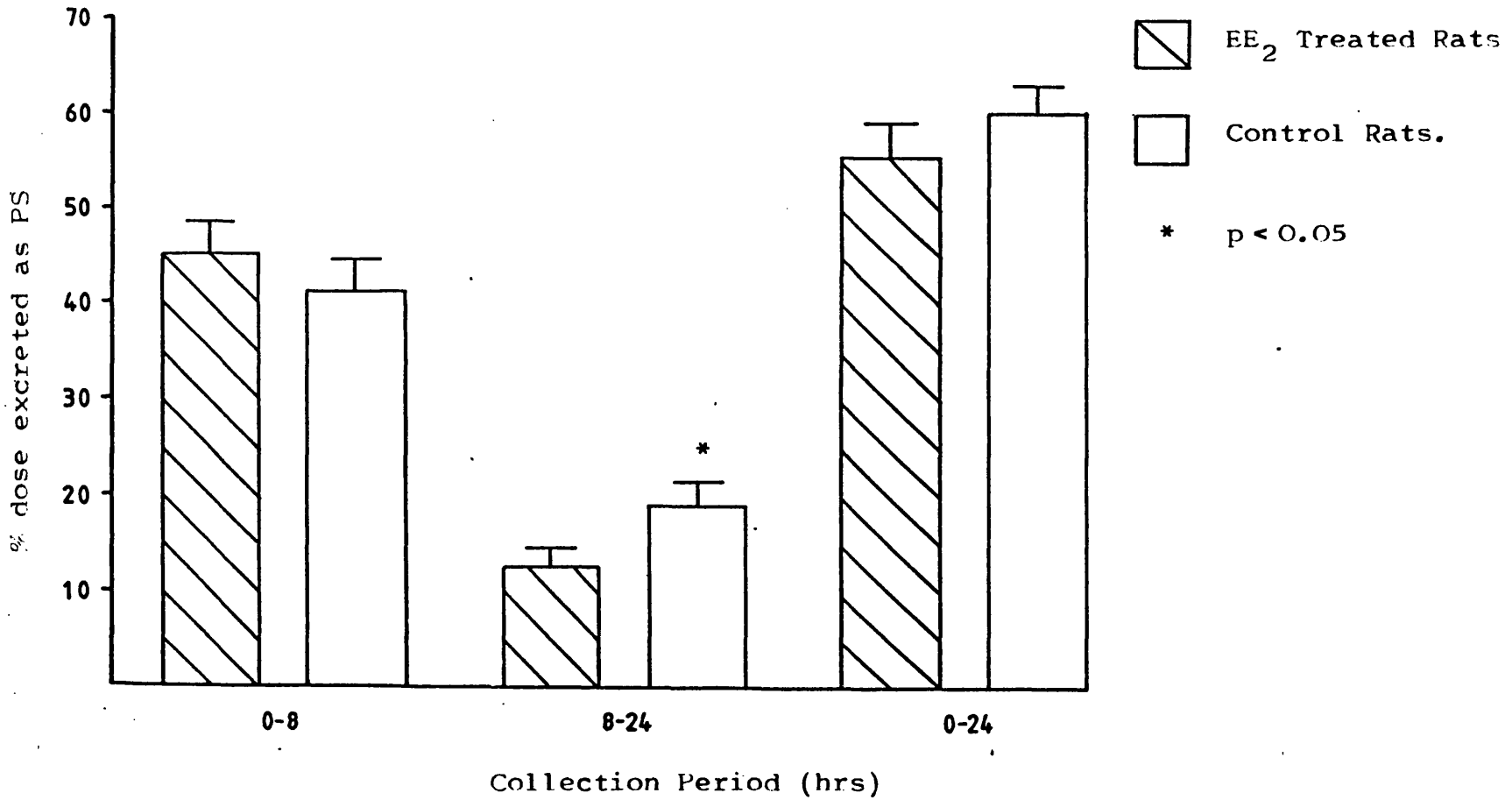


FIGURE 6.4

The urinary excretion of P + PG + PS in female rats.

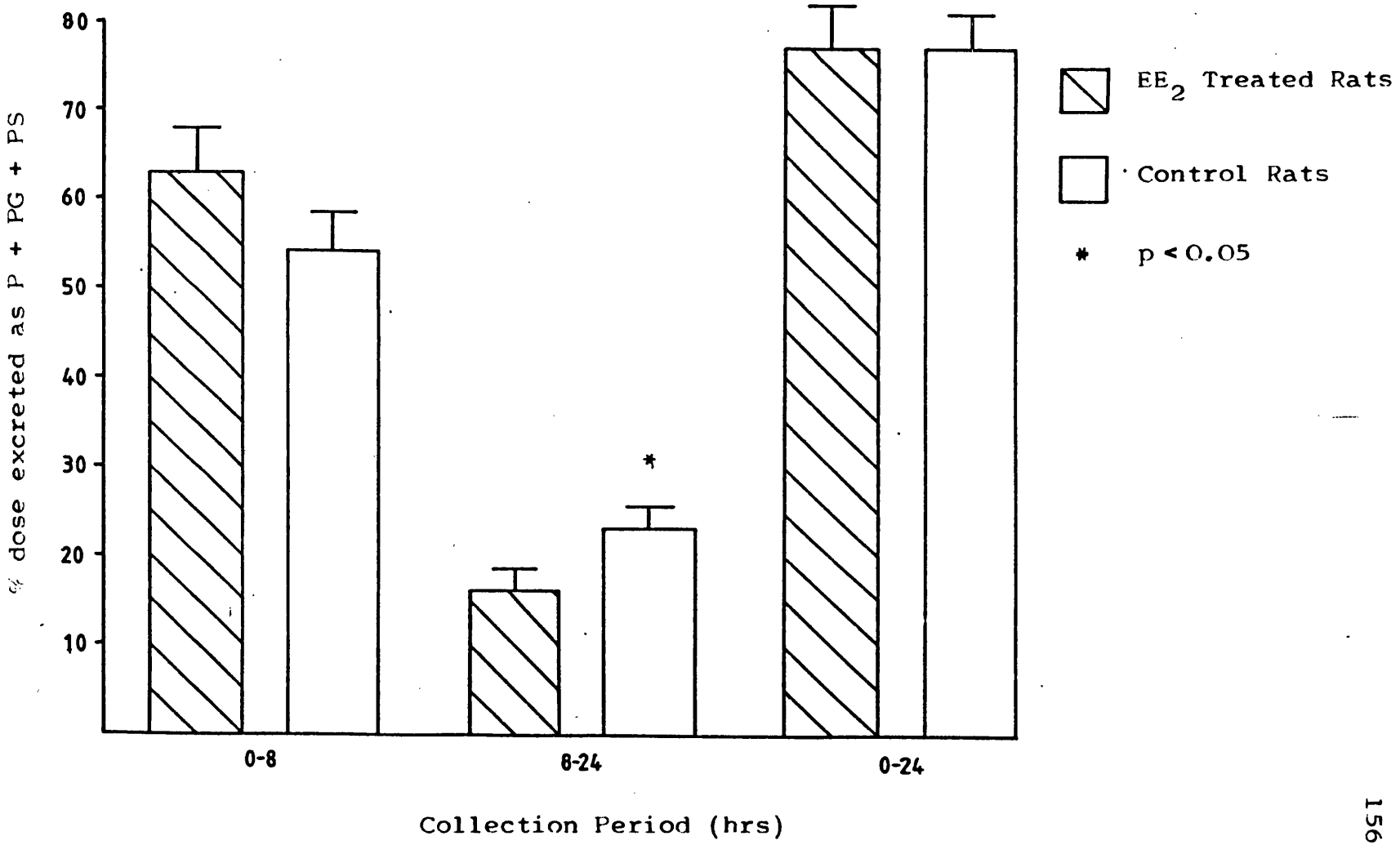


Table 6.2. Serum paracetamol clearance from 0 to 8 hours

(Mean ± SE)

Mins	PARACETAMOL IN SERUM (µg/ml)	
	TEST	CONTROL
10	12.0 ± 0.9	13.9 ± 1.9
15	24.3 ± 2.2	32.1 ± 0.9
30	6.0 ± 0.2	7.0 ± 1.1
45	10.3 ± 1.2	12.9 ± 1.0
60	4.0 ± 0.06	5.3 ± 0.6
90	4.8 ± 0.6	5.6 ± 0.4
120	3.0 ± 0.4	3.8 ± 0.8
180	1.95 ± 0.13	2.04 ± 0.16
210	0.48 ± 0.03	0.70 ± 0.05
240	0.76 ± 0.06	1.02 ± 0.03
360	0.48 ± 0.04	0.68 ± 0.15
480	0.345 ± 0.045	0.54 ± 0.12
AUC	1092.8 µg/ml	1412.8 µg/ml
$t_{½}\alpha$	51.9 min	57.9 min
$t_{½}\beta$	301.3 min	385.0 min

FIGURE 6.5a

Paracetamol serum clearance in female rats from 0 to 3 hours.

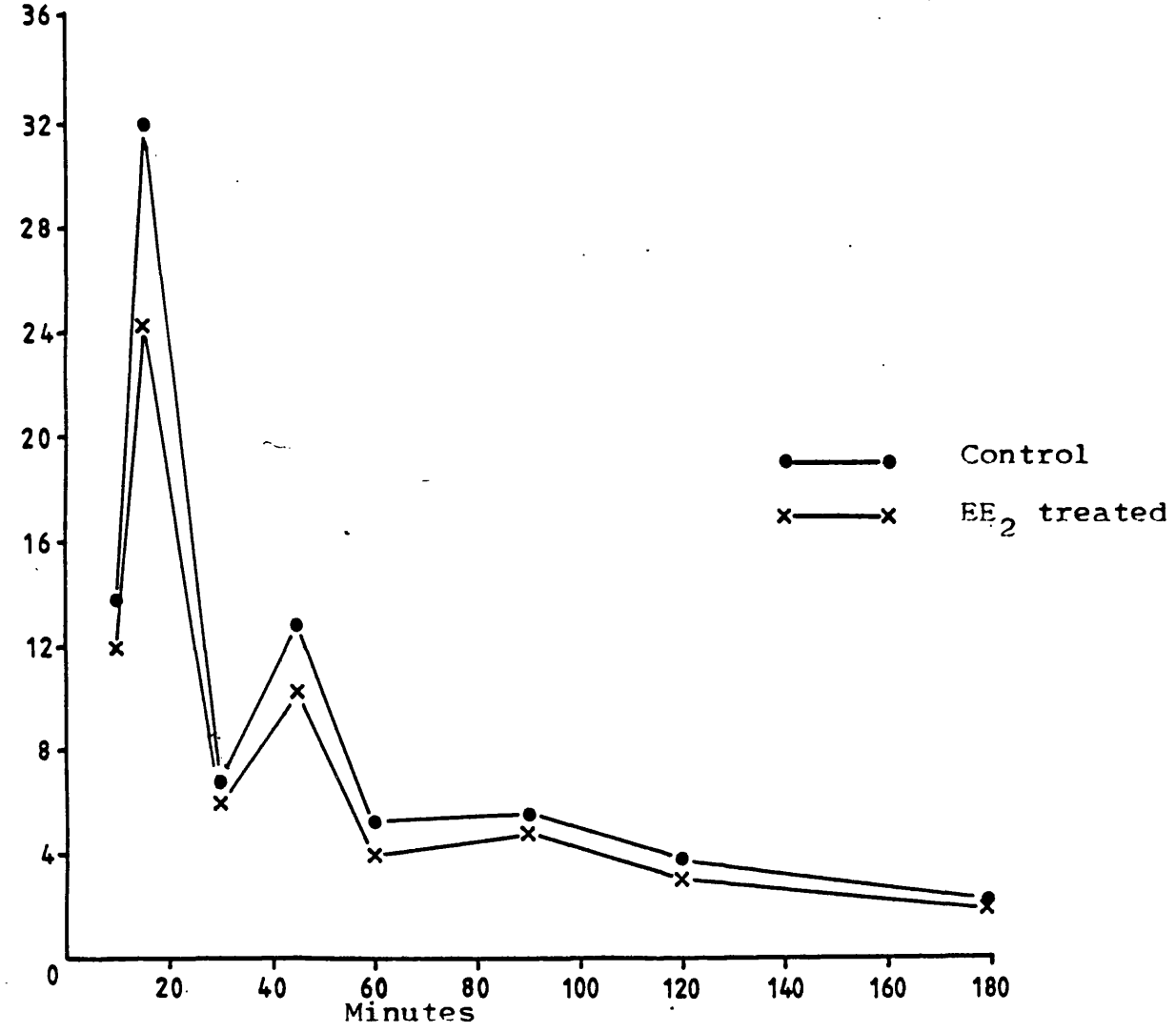
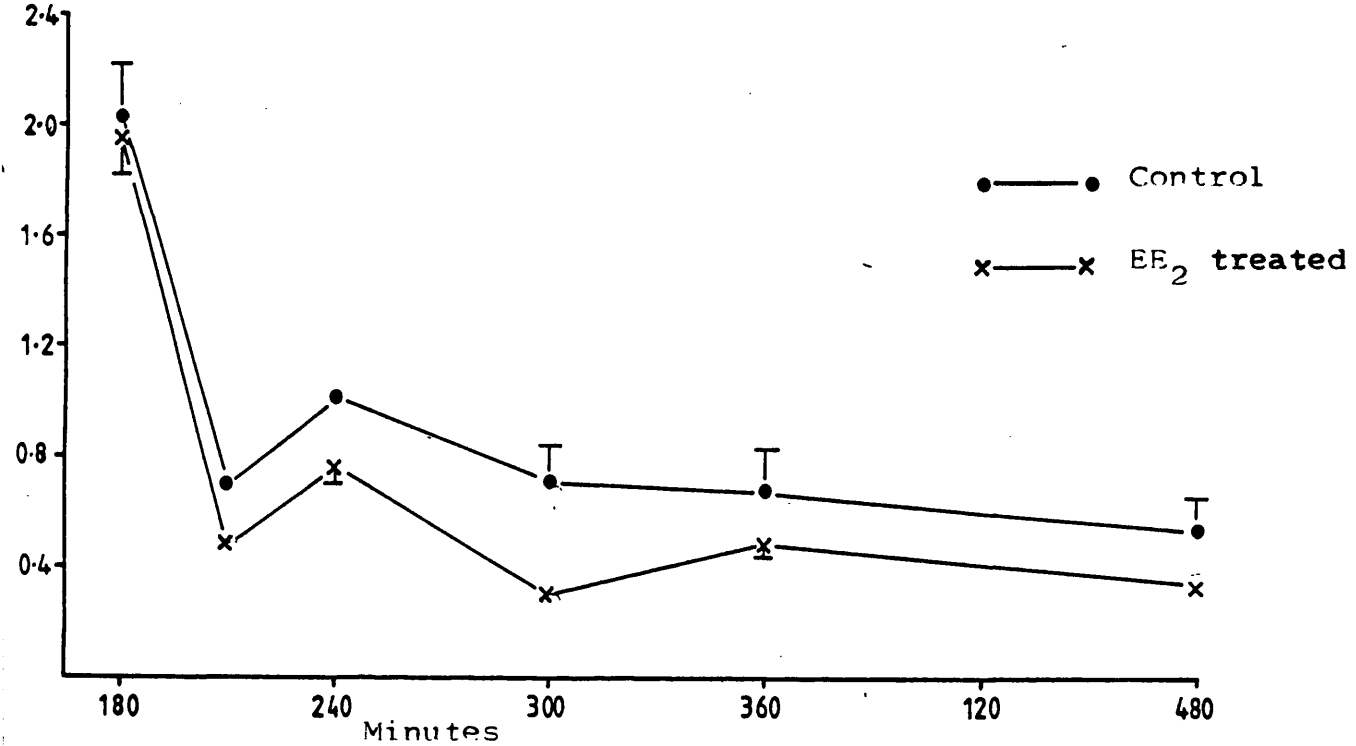


FIGURE 6.5b

Paracetamol serum clearance in female rats from 3 to 8 hours.



The mean serum paracetamol concentration was found to be lower in the EE_2-treated rats than the control rats at all time points, the difference becoming greater after 3 hours. However, possibly due to the interanimal variation and the small number of animals used per time point, these differences in serum concentration failed to reach significance. The area under the time-concentration curve (AUC) appeared to be lower in the treated animals. Furthermore, the elimination half-life ($t_{½}\beta$) also appeared to be lower in the test rats, although similar values were obtained for the initial half-life ($t_{½}\alpha$) for both the test and control rats.

b) Discussion

The present study has shown that chronic pretreatment with EE_2 results in a significant increase in the 0 to 8 hour urinary excretion of PG, suggesting that the glucuronidation pathway has been induced. As PG is not the major metabolite of paracetamol in the rat the total dose recovered was not significantly altered by EE_2 pretreatment. However, both the $AUC_{(0-8\ hr)}$ and the half-life, $t_{½}\beta$, appeared to be lower in the EE_2-treated rats which suggests that the serum clearance of paracetamol may be increased by EE_2 pretreatment. Therefore, the inductive effect of EE_2 on paracetamol glucuronidation found in the rat may be sufficient to cause an apparent increase in paracetamol serum clearance, together with an apparent decrease in paracetamol half-life.

Combined OCS containing EE_2 have been found to cause induction of the glucuronide pathways in females using this form of hormonal

contraception. Drugs such as paracetamol, clofibric acid (Miners *et al.*, 1983, 1984, rspectively); lorazepam and oxazepam (Patwardhan *et al.*, 1981) have been reported to show induction of glucuronidation in chronic OCS users. Similarly the present study has indicated that OCS use results in an increase in PG excretion as discussed in Chapter 4. The findings of the present rat study suggests that it is the EE_2 component of the OCS which is responsible for the induction of glucuronidation found in the several human studies reported earlier.

In contrast, Hargreaves *et al.* (1971) found that EE_2 reduced the glucuronide conjugation of bilirubin and o-aminophenol when studied *in vitro*. However, Watanabe (1970) reported that pre-treatment with EE_2 resulted in a decreased bile flow ($p < 0.001$) and Hiekel and Lathe (1970) found that OCS caused a decrease in bilirubin excretion. Therefore, the results of Hargreaves *et al.* may be due to a decrease in bilirubin excretion rather than a decrease in bilirubin conjugation. Furthermore, the study of Hargreaves *et al.* was performed *in vitro* using rat liver slices and no pretreatment with EE_2 occurred. Hence, the results found are not directly comparable with the present study where the rats were pre-treated for up to 40 days in an attempt to mirror the normal clinical situation of OCS use.

Although in the present study, EE_2 pretreatment was found to cause induction of paracetamol glucuronidation, a delayed inhibition of paracetamol sulphation was also found. In the 8 - 24 hour urine, significantly less PS was excreted, and as this is the major metabolite in the rat, this resulted in a significant decrease in

the percentage recovery of the drug in this time period. If the glucuronidation and sulphation pathways of paracetamol were in competition with each other, as found in the human paracetamol study as discussed in Chapter 4, the induction of paracetamol glucuronidation could account for the decrease in paracetamol sulphation. However, the delayed decrease in PS excretion could also be due to depletion of available sulphate as both EE_2 and paracetamol undergo sulphate conjugation and so may be in competition with each other for the supplies of sulphate. Furthermore, the 2 compounds may be in direct competition for the enzyme sulphotransferase, and as the concentration of paracetamol becomes progressively lower, EE_2 may competitively inhibit the sulphation of paracetamol. No work has been published to date on the effect of EE_2 on sulphate conjugation, and so further studies have to be performed to determine how EE_2 pretreatment affects sulphate metabolism.

6.4 ESTRADIOL STUDY

a) Results

Table 6.3 and Fig. 6.6 show the serum concentrations of estradiol for both control rats and the EE_2-treated rats. From 1 to 16 minutes the serum estradiol concentrations were found to be higher in the control rats than in the test rats; however, from 20 to 120 minutes the reverse was true, estradiol concentrations were higher in the test rats than in the control animals. Possibly due to the large inter-animal variation and the small number of animals used per time point, these differences failed to reach significance. The area under the time-concentration curve (AUC_{1-150}) was similar in both test and control rats (30.04×10^3 and 29.02×10^3 DPM/0.1 ml min respectively). Also the initial half-life ($t_{1/2}\alpha$) and the elimination half-life ($t_{1/2}\beta$) appeared to be similar in both animal groups, with the control rats having half-lives of 6.1 and 53.7 min and the test rats having half-lives of 5.4 and 49.5 min.

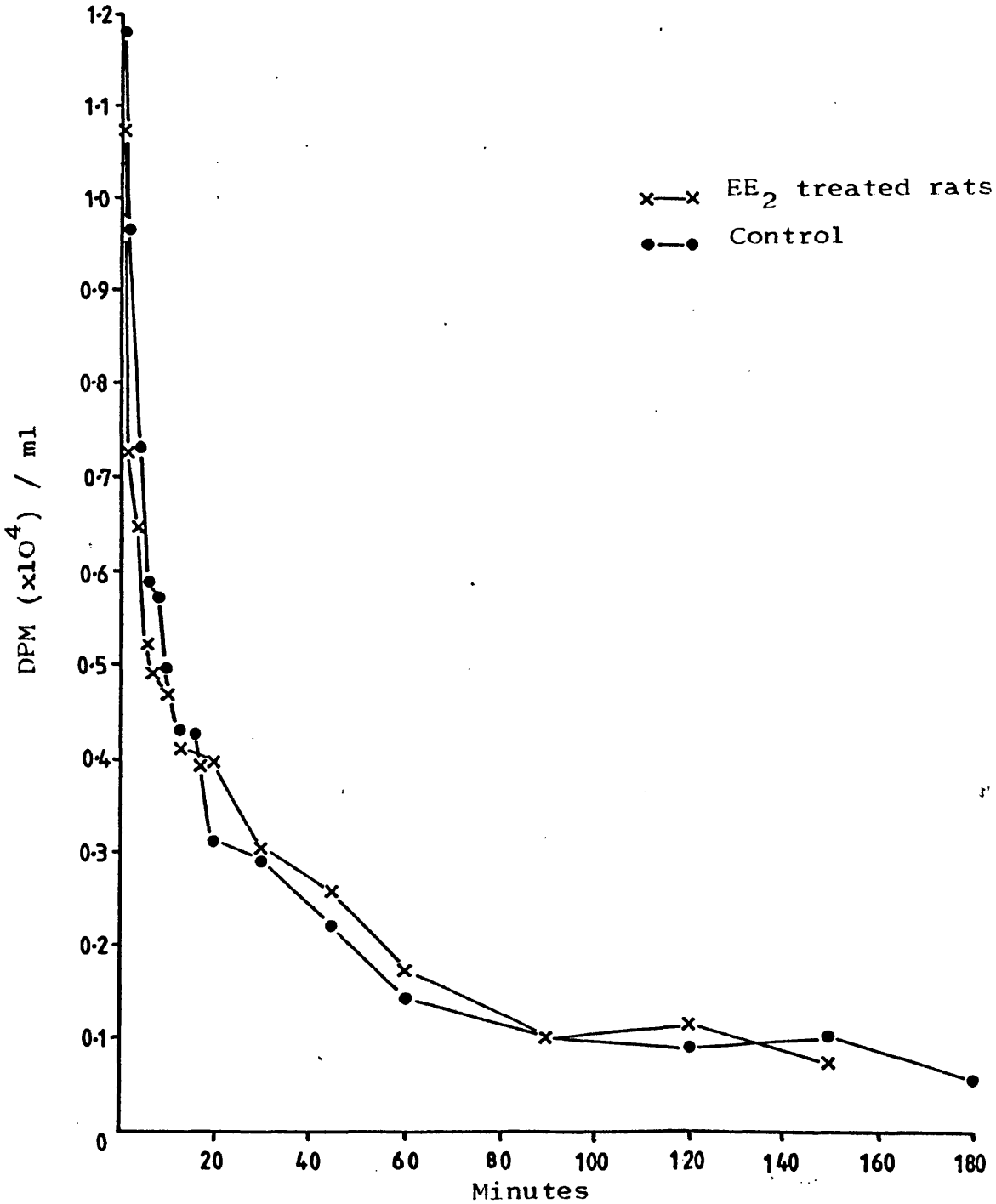
The 24-hour urinary excretion of estradiol, together with the tissue content of administered estradiol for both control and treated rats is given in Table 6.4 and Figs. 6.7 to 6.9.

The 24-hour urinary excretion of total estradiol was found to be significantly higher in the EE_2-treated rats than in the control animals ($p < 0.01$) whilst the radioactive content of the liver was also apparently lower in EE_2-treated rats, but possibly due to the inter-animal variation this difference failed to reach significance ($p < 0.1$). Similarly, the radioactivity found in the large

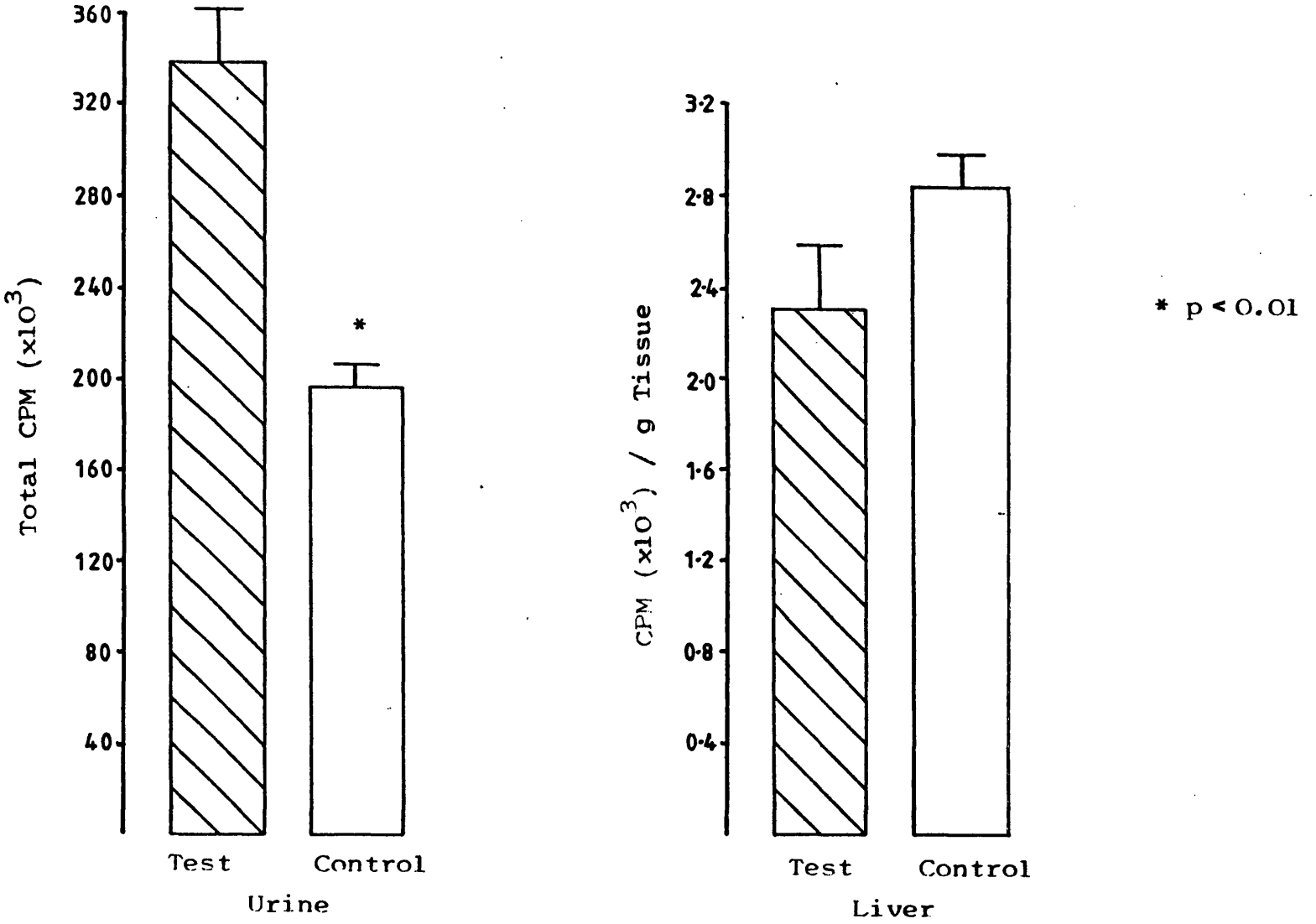
Table 6.3. Serum estradiol clearance from 0 to 3 hours

(Mean ± SE)

TIME (minutes)	Serum concentration of $^{total}_{\Lambda}$estradiol (DPM/0.1 ml) CONTROL	TEST
1	1117.8	1074.5 ± 205.2
2	963.1 ± 42.1	757.95 ± 163.0
4	666.75 ± 97.2	644.5 ± 120.3
6	588.1 ± 65.8	605.0
8	572.0 ± 96.5	489.6 ± 55.6
10	495.4 ± 41.3	472.4 ± 34.1
13	432.5 ± 29.75	412.6 ± 36.7
16	426.3 ± 34.1	389.6 ± 9.6
20	308.6 ± 24.9	396.2 ± 17.7
30	287.8 ± 21.2	299.9 ± 25.4
45	212.4 ± 24.4	320.2 ± 68.0
60	137.3 ± 9.7	170.25 ± 12.2
90	98.5 ± 19.25	101.8
120	88.5 ± 7.3	115.25
180	54.0 ± 8.0	
AUC	29.02×10^3 DPM/0.1 ml	30.04×10^3 DPM/0.1 ml
$t_{1/2}$	5.1 min	5.4 min
$t_{1/2}$	53.7 min	49.5 min


1·2
1·1
1·0
0·9
0·8
0·7
0·6
0·5
0·4
0·3
0·2
0·1
0
DPM (x10⁴) / ml
20
40
60
80
100
120
140
160
180
Minutes
EE2 treated rats
Control

^{14}C- estradiol content of 0 to 24 hr. urine and liver



^{14}C- estradiol content of individual intestinal tissues.

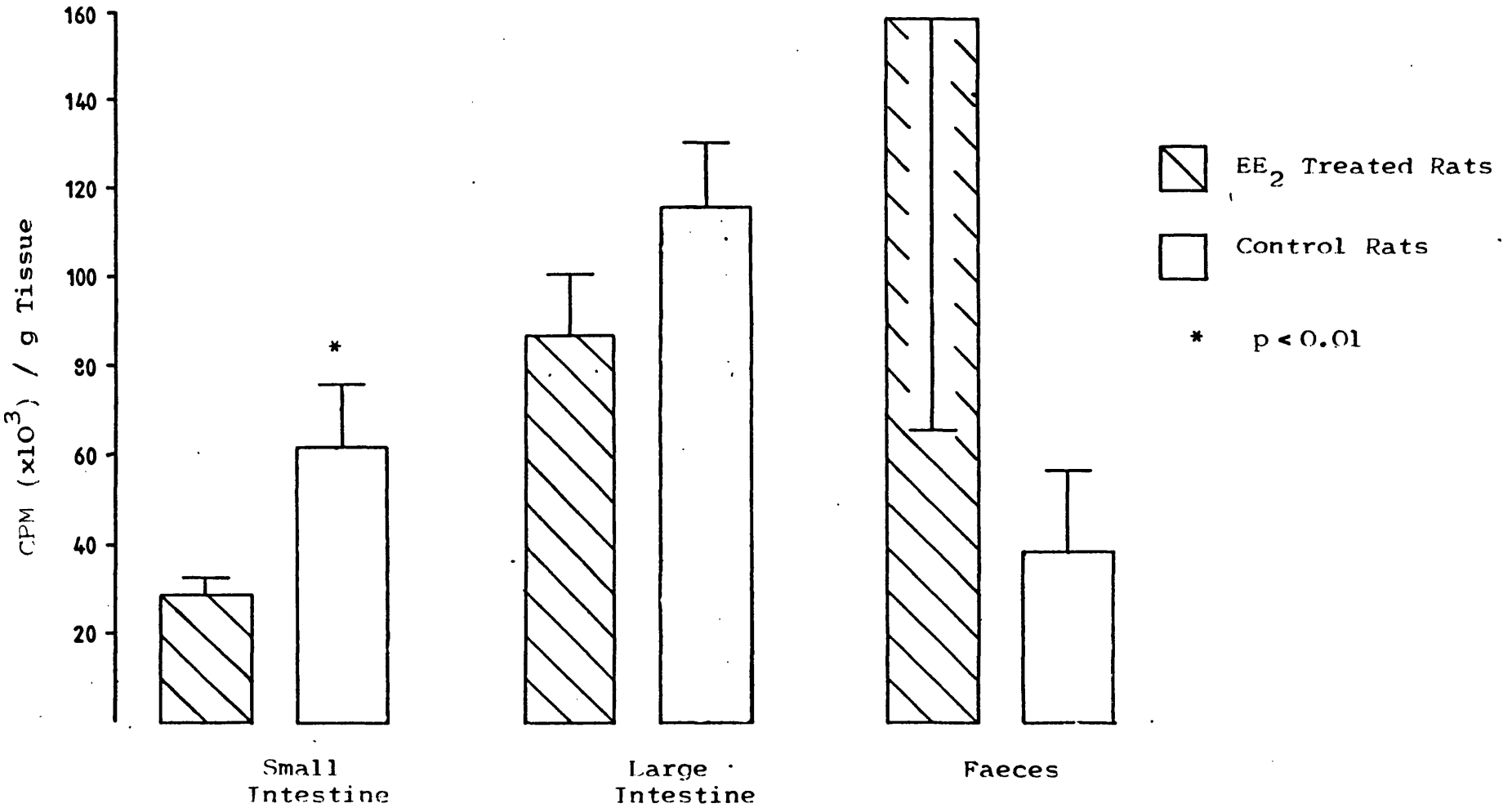


FIGURE 6.9

^{14}C- estradiol content of combined intestinal tissues.

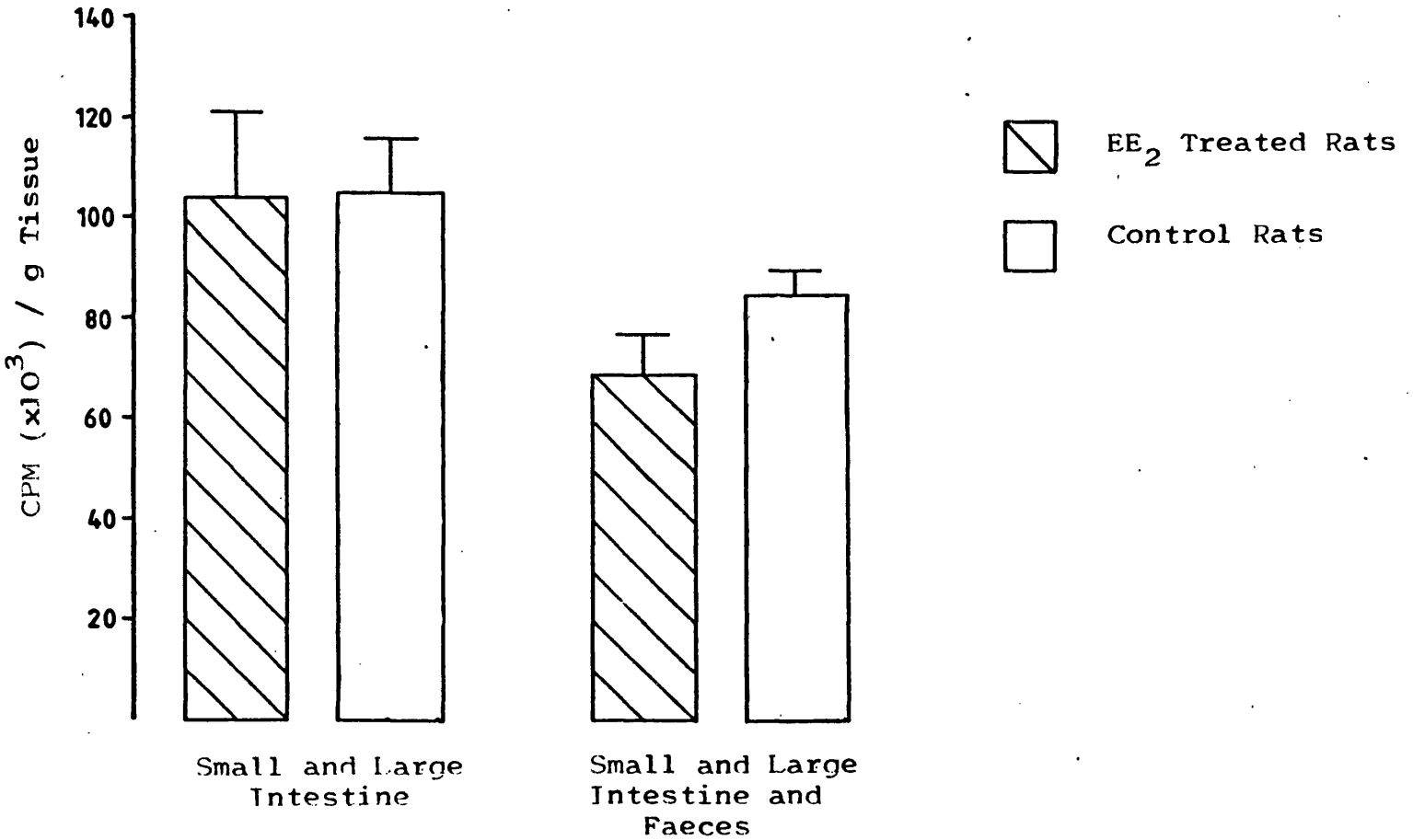


Table 6.4. The 24-hr urinary and tissue estradiol content in test and control rats (mean ± SE)

TISSUE	ESTRADIOL CONTENT (CPM ($x10^3$)/g tissue)	
	TEST (n=5)	CONTROL (n=5)
Liver	2.31 ± 0.28	2.84 ± 0.14
Small intestine (SI)	28.66 ± 4.20*	62.28 ± 14.28
Large intestine (LI)	86.66 ± 14.07	116.10 ± 14.63
Faeces	158.86 ± 92.98	39.33 ± 18.32
Faeces + LI	103.61 ± 17.28	105.21 ± 10.81
Faeces + LI + SI	68.82 ± 7.80	84.85 ± 4.90
Urine	33.77 ± 2.38* CPM	19.69 ± 1.02 CPM

* $p < 0.01$ compared to control.

intestine and the faeces showed no significant difference between the test and control rats ($p < 0.1$). However, the radioactive content of the small intestine after 24 hours was found to be significantly lower in the test rats ($p < 0.01$).

b) Discussion

The chronic administration of EE_2 to female rats resulted in slight apparent differences in the serum concentrations of estradiol. However these apparent differences were not reflected in the pharmacokinetic variables, area under the time-concentration curve (AUC) and the half-lives. The AUC together with the initial and

elimination half-lives for both test and control rats were similar indicating a similar serum clearance for estradiol in the 2 animal groups. Hence chronic treatment with EE_2 appears to have no effect upon the initial (0 - 3 hour) clearance of estradiol. This is in contrast to the results obtained by Slikker *et al.* (1983), where chronic use of a combined oral contraceptive containing ethynerone and mestranol was found to significantly increase estradiol clearance from 0 - 6 hours. This discrepancy could be due to species differences as Slikker *et al.* used monkeys whereas rats were used in the present study. Furthermore, Slikker *et al.* administered a progestin, ethynerone, and the estrogen mestranol, whereas the estrogen, ethinylestradiol alone was administered in the present work. Also the time period used by Slikker *et al.* to determine estradiol clearance was double that used in the present study.

Although chronic EE_2 pretreatment was found to have no effect upon the 0 to 3 hour serum clearance of estradiol, the 24-hour urinary excretion of estradiol was significantly increased in the EE_2-treated rats ($p < 0.01$). As estradiol is subject to enterohepatic circulation, the estradiol content of the liver, small intestine, large intestine and faeces, 24 hours after administration were also studied. The liver appeared to have a lower estradiol content in the treated rats when compared to the control group, but possibly due to the interanimal variation, this failed to reach significance. The estradiol content of the faeces and the large intestine showed no significant difference between the 2 groups but the estradiol content of the small intestine was significantly lower in the EE_2-treated rats. Thus the present study

has shown that chronic use of EE_2 in female rats significantly increases the 24-hour urinary excretion of estradiol whilst there was significant lowering of the estradiol content of the small intestine determined 24 hours after ^{14}C-estradiol administration. These results suggest that the increase in the urinary output of estradiol may be due to the decrease in the intestinal content. Furthermore, the decreased estradiol content of the small intestine indicates that EE_2 has some effect upon the enterohepatic circulation of estradiol.

Chronic pretreatment of rats with EE_2 at doses of 0.5 to 1.0 mg/kg for 5 to 9 days has been shown to decrease bile flow and biliary excretion (Heikel and Lathe, 1970; Kreek *et al.*, 1969; Watanabe, 1971). SImilarly acute treatment with the glucuronide conjugate of EE_2 has also been found to decrease bile flow (Vore *et al.*, 1983). Therefore the reduction in bile flow caused by EE_2 may result in decreased biliary excretion of estradiol and so could account for the decreased estradiol content of the small intestine found in the EE_2-treated rats of the present study.

Furthermore, if bile flow was decreased, resulting in decreased biliary excretion of estradiol, this would result in increased concentrations of estradiol leaving the liver. However only approximately 2% of the administered dose undergoes biliary excretion as free estradiol; the remainder is excreted into the bile as glucuronide conjugates, the major metabolites being the glucuronide conjugates of 2-methoxyestrone and 2-hydroxyestrone (Watanabe, 1970). Thus, the decreased bile flow causes a decrease

in the biliary excretion of the glucuronide conjugates. This results in the concentration of the estradiol glucuronides leaving the liver being raised and as these compounds are highly water-soluble, they are readily excreted in the urine.

The increased urinary excretion of estradiol, found in EE_2-treated rats may be due to decreased enterohepatic circulation of estradiol, as indicated by the lowered total estradiol content of the small intestine. In addition the increased urinary excretion of estradiol may also be due to induction of estradiol metabolism. Chronic use of EE_2 has been shown to induce paracetamol glucuronidation in female rats (Chapter 6.3) and so it may also cause induction of the glucuronidation of estradiol. Following chronic administration of combined OCS to monkeys, Slikker *et al.*, (1984), found that the clearance of exogenously administered estradiol was significantly increased in the treated animals, which they attributed to the increased urinary excretion of estrone and estrone glucuronide. Therefore, Slikker *et al.* stated that the OCS used caused induction of estradiol metabolism namely the biotransformation to estrone and the glucuronidation pathway. Hence, combined OCS use has been shown to induce estradiol metabolism in monkeys and the present study indicates that EE_2 pretreatment may also result in induction of estradiol metabolism.

CHAPTER SEVEN

COMPARISON OF FACTORS AFFECTING GLYCINE, GLUCURONIDE AND SULPHATE CONJUGATION

7.1 GENETIC CONTROL OF DRUG METABOLISM

Population studies involving the administration of a probe drug and the determination of its metabolic profile are widely used in order to examine the genetic control of various metabolic biotransformations. One of the earliest examples of this form of pharmacogenetic study was performed by Evans *et al.* (1960), whereby the 8-hour urinary excretion of N-acetyl isoniazid, the major metabolite of the probe drug isoniazid, was determined in a large number of subjects. A bimodal distribution was found, indicating that N-acetylation of isoniazid is subject to genetic control with approx. 38% of the UK population studied being slow acetylators. Family studies were then performed and slow acetylation was found to be an autosomal recessive trait. Other compounds which also undergo acetylation have been found to be subject to genetic control; these included dapsone, hydralazine, procainamide, phenelzine, nitrazepam and certain sulphonamides (for references see Lunde *et al.* (1977)).

A significant progress was made in the field of pharmacogenetics during the 1970's when Mahgoub *et al.* (1977) discovered monogenic control of oxidation. The approach was similar to that used by Evans *et al.* and involved the determination of the 0 - 8 hr urinary excretion of the probe drug debrisoquine and its major metabolite

4-hydroxydebrisoquine. The metabolic ratio between debrisoquine and 4-hydroxydebrisoquine gave a bimodal distribution with approximately 3% of the population studied being poor metabolisers and family studies showing that the poor oxidiser phenotype is controlled by an autosomal recessive gene. At least 22 other drugs which utilise the same hepatic drug oxidising pathway as debrisoquine have been found to be subject to genetic control including sparteine (Eichelbaum *et al.*, 1979); phenytoin (Sloan *et al.*, 1981); certain β-blockers (Dayer *et al.*, 1985); guanoxan and phenacetin (Sloan *et al.*, 1978).

Oxidation is the major phase I metabolic pathway and as a result numerous pharmacogenetic studies have been and still are being performed for the wide range of drugs which undergo this biotransformation. However, acetylation is only one out of the 8 phase II metabolic reactions which are known to occur in man. Very few pharmacogenetic studies have been performed on the other 7 conjugation pathways. Therefore in the present work, population studies were carried out to determine whether glycine, glucuronide and sulphate conjugation were subject to genetic control using aspirin and paracetamol as the probe drugs. The 0 - 8 hour urinary excretion of salicylic acid (SA) and its major metabolite, the glycine conjugate, salicyluric acid (SUA) was determined in 150 healthy volunteers following an oral therapeutic dose of aspirin (600 mg). Similarly, the 0 - 8 hour urinary excretion of paracetamol (P) and its major metabolites, paracetamol glucuronide (PG) and paracetamol sulphate (PS) was determined in 99 healthy

volunteers following an oral therapeutic dose of paracetamol (1 g). In the population study for aspirin discussed in Chapter 3, the metabolic ratio between SA and its major metabolite, SUA, showed a skewed distribution. However, together with the large interindividual variation found for the metabolic ratio SA/SUA a large intra individual variation was also found. The skewed distribution indicates that there may be genetic control of the glycine conjugation of SA, however as the distribution was not multi-modal and no separate phenotypes could be distinguished, environmental factors may have a large influence on salicylate metabolism. Furthermore the large intraindividual variation of the salicylate metabolic ratio shows that environmental factors probably do have a greater influence on the glycine conjugation of SA than genetic factors. In the population study for paracetamol discussed in Chapter 4, the metabolic ratios between P and PG and between P and PS both showed unimodal distributions, indicating that the glucuronide and sulphate conjugation of paracetamol are subject to polygenic and multifactorial control. In contrast, the large numbers of drugs which undergo oxidation and acetylation both show bimodal distributions with 2 distinct phenotypes, together with small intraindividual variations, indicating strong monogenic control with environmental factors having only a comparatively weak effect on these 2 metabolic pathways.

Genetic control of phase I metabolism has been extensively studied and a large number of drugs which undergo oxidation have been found to be subject to monogenic control. However, although phase II

metabolism is responsible for terminating the activity of many exogenous and endogenous compounds, the study of genetic control in conjugation reactions has been limited to acetylation, which is also known to be under monogenic control. Therefore, the present work was undertaken to extend the knowledge of genetic control of phase II metabolism. The population study for paracetamol provided no evidence for the genetic control of sulphate and glucuronide conjugation whilst the population study for aspirin showed glycine conjugation may be subject to weak genetic control, but environmental factors have a far greater influence. Hence, three of the major phase II reactions; glycine, glucuronide and sulphate conjugation are not subject to genetic control.

7.2 ENVIRONMENTAL CONTROL OF PHASE II METABOLISM

a) Age

The present work has shown that age has no effect upon the glycine conjugation of SA nor upon the glucuronide and sulphate conjugation of paracetamol in the age ranges studied of 18 to 61 years and 18 to 62 years respectively. Conversely, other workers have found that the renal clearance of SUA decreases with increasing age (e.g. Ho *et al.*, 1985) and paracetamol plasma clearance has been found by several workers to be significantly decreased in elderly subjects (e.g. Triggs *et al.*, 1975). The contrasting results obtained from these studies compared to those of the present work are probably due to the limited age range of the latter, for both Ho *et al.* and Triggs *et al.* included subjects aged over 65 years whilst the studies discussed in Chapters 3 and 4 had a maximum age of 61 and 62 years respectively. Therefore, in order to detect any effect of age upon drug metabolism, subjects aged 65 or over must be included in the study.

Although it was not found in the present work due to the limited age range used, other workers have shown that old age (i.e. 65 years or over) appears to decrease the capacity for the phase II metabolic pathways; glycine, glucuronide and sulphate conjugation. The major phase I metabolic pathway, hepatic oxidation, has also been found to be significantly lowered in elderly subjects (Crooks and Stevenson, 1977). Hence it would seem that aspects of both phase I and phase II metabolism are impaired in elderly subjects aged over 65 years.

b) Gender

The aspirin study discussed in Chapter 3 showed that females excreted significantly less SUA and significantly more SA than males following aspirin administration. Several workers have also reported that the plasma clearance of salicylate is significantly decreased in females (Ho *et al.*, 1985; Miners *et al.*, 1986) which, as the present work has shown, is due to a decreased capacity for glycine conjugation.

The paracetamol study discussed in Chapter 4 showed no sex differences in the urinary excretion of P, PG and PS. This is in contrast to other workers who have found decreased paracetamol plasma clearance in females, accompanied by a decreased renal and metabolic clearance of PG (Miners *et al.*, 1983), which indicates a decreased capacity for glucuronide conjugation in females. However, as no sex differences were found in paracetamol metabolism in the present work, which involved a large number of volunteers, the decreased paracetamol clearance found in females by other workers, which involved a comparatively small number of volunteers, may be of little clinical significance.

Recent studies have shown sex differences in oxidative metabolism with female rats showing pronounced decreased clearance of a number of drugs which undergo oxidation (Kato, 1974). Similarly, studies in man have shown that females have a decreased oxidative capacity compared to males, as indicated by the decreased clearance of drugs such as diazepam (Geenblatt *et al.*, 1980) and chlordiazepoxide (Roberts *et al.*, 1980) found in females. Thus, the phase I metabolic reaction, oxidation, and the phase II reactions, glycine

and possibly glucuronide conjugations, have been shown to be sex dependent, with females having a reduced capacity for these pathways, whilst sulphate conjugation appears to show no sex differences.

c) Smoking

Smoking was found to have no effect upon the glycine conjugation of SA, nor upon the sulphate conjugation of paracetamol in the present work. However, the glucuronide conjugation of paracetamol was found to be significantly increased by smoking, although this effect was found in females only.

Cigarette smoke contains polycyclic hydrocarbons (Wynde and Hoffman, 1950) which are known to be potent enzyme inducers. The activity of several hepatic microsomal enzyme systems is increased by these compounds including N-demethylation, hydroxylation, reduction and glucuronide conjugation (Conney and Burns, 1962), although considerable substrate specificity is exhibited.

The enzyme systems subject to induction by polycyclic hydrocarbons are believed to be situated in the hepatic microsomes (Conney and Burns, 1962) and both glycine and sulphate conjugation are non-microsomal (glycine conjugation occurring in the hepatic mitochondria and sulphation occurring in the kidney, intestinal mucosa and the soluble fraction of the hepatic cell). Therefore, it is to be expected that these 2 metabolic pathways are not affected by cigarette smoking. Conversely, glucuronidation is a hepatic microsomal pathway, and the results of the present study indicate that paracetamol is a suitable substrate for induction by the

polycyclic hydrocarbons present in cigarette smoke. Hence it would appear that cigarette smoke induces a variety of phase I metabolic reactions, but glucuronidation may be the only phase II reaction which it is known to induce.

d) Oral Contraceptive Steroids

In the present work, oral contraceptive steroids were found to have no effect upon the glycine conjugation of salicylate. This is in contrast to the results of other studies performed in small groups of subjects where the plasma clearance of salicylate was found to be increased in OCS users (Gupta *et al.*, 1982; Miners *et al.*, 1986) indicating an increased glycine conjugation in these subjects. However, due to the small number of subjects used and the near basal conditions employed, this induction may be of little clinical significance.

The glucuronidation of paracetamol was found to be increased in OCS users in the present study, although this difference failed to reach significance whereas the sulphation of paracetamol was found to be significantly decreased by OCS use. As glucuronidation and sulphation of paracetamol have been shown to be in competition with each other (see Chapter 4), a decrease in one pathway will result in an increase in the competing pathway and vice versa, and so the increase in glucuronidation found in OCS users may be the cause of the decrease in sulphation.

Several other workers have found paracetamol plasma clearance to be decreased by OCS use (Miners *et al.*, 1983; Mitchell *et al.*, 1983). This increase in paracetamol clearance was reported to be due to

induction of the glucuronidation pathway with the OCS having no effect upon the sulphate conjugation.

The estradiol study discussed in Chapter 5 found significant increases in the urinary metabolic ratios E_2/E_2G and E_2/E_2S in the subjects using OCS, which indicated that OCS may decrease the oxidation of estradiol. This result is supported by a study performed by Femino *et al.* (1974), which stated that OCS use decreased the urinary excretion of the 16 -hydroxylated metabolites of exogenously administered estradiol. Furthermore, numerous other compounds which undergo oxidation, have also shown a decreased plasma clearance in OCS users, including antipyrine (Abernethy and Greenblatt, 1981); chlordiazepoxide (Roberts *et al.*, 1979) and caffeine (Patwardhan *et al.*, 1981).

In addition to the possible effects on the oxidation pathway, the estradiol study of the present work has also shown a significant increase in the metabolic ratio E_2G/E_2S in OCS users, indicating either a possible increase in estradiol glucuronidation or a possible decrease in estradiol sulphation. Several workers have found that OCS use results in induction of glucuronidation of several drugs including paracetamol and clofibric acid (Miners *et al.*, 1983, 1984); lorazepam and oxazepam (Patwardhan *et al.*, 1981). No reports have been published on OCS causing decreased sulphation, apart from the paracetamol study discussed in Chapter 3, where glucuronidation and sulphation were competing pathways. Hence chronic OCS use may cause induction of endogenous estradiol glucuronidation rather than inhibition of estradiol sulphation.

Animal Studies: Effect of EE_2 on Phase II Metabolism

The chronic pretreatment of EE_2 in female rats as discussed in Chapter 6, has been found significantly to increase the 0 - 8 hour PG urinary excretion followed by a delayed significant decrease in the 8 - 24 hour urinary excretion of PS. An apparent increase in paracetamol plasma clearance was also obtained in the EE_2-treated rats, although this difference failed to reach significance. Hence, chronic administration of EE_2 appears to cause induction of the glucuronidation pathway which may be responsible for the apparent increased plasma clearance. The decrease in PS excretion observed in EE_2-treated rats may be either due to competition between EE_2 and paracetamol for the sulphotransferase enzyme or the available supply of sulphate. An alternative explanation may be that sulphation and glucuronidation of paracetamol may be in competition with each other as found in man (see Chapter 4) and so an increase in PG excretion will result in a subsequent decrease in PS excretion.

The induction of the glucuronidation pathway of paracetamol due to EE_2 pretreatment, mirrors the situation found in man due to OCS use. Hence the estrogenic component of the OCS may be responsible for this induction although the studies performed in man and the rat study discussed in Chapter 5 are not directly comparable. This is due to the rats receiving a much larger daily dose of EE_2 (11 µg per day) on a mg/kg basis, than the daily dose of EE_2 taken during combined OCS use (30 or 50µg per day) by females. Large doses of EE_2 were given to the rats in order to determine whether EE_2 pretreatment could competitively inhibit the conjugation of paracetamol, as

both compounds undergo glucuronide and sulphate conjugation. The present work has indicated that it is the estrogenic component of combined OCS which causes induction of glucuronide conjugation. Therefore, if a female using combined OCS (containing EE_2) takes another drug concomitantly, which is inactivated by glucuronidation, the plasma clearance of the second drug may be increased, resulting in a decreased activity of that drug.

The effect of chronic EE_2 pretreatment on estradiol metabolism was also determined using female rats. The 0 - 24 hour urinary excretion of total estradiol was found to be significantly increased by EE_2 pretreatment, whilst the small intestine content of total estradiol was found to be significantly lowered. Therefore, it was suggested that the increased urinary excretion of total estradiol may be due to decreased enterohepatic circulation. OCS have been found to reduce the bile flow and decrease biliary excretion (Heikel and Lathe, 1970; Kreek *et al.*, 1969) and as conjugates of estradiol and conjugates of its phase I metabolites, estrone and estriol, are excreted into the bile, a decrease in bile flow will result in a subsequent decrease in the biliary excretion of the estrogen conjugates. Hence decreased biliary excretion of the estrogen metabolites could account for the increased urinary excretion of total estradiol. Furthermore, EE_2 may be causing induction of the glucuronidation pathway, as was found in the paracetamol rat study (see Chapter 6.3). This induction will further enhance the increased urinary excretion of total estradiol. The present work has indicated that chronic OCS use in females may induce the glucuronidation of estradiol (see Chapter 5), and the

increased urinary excretion of estradiol found in the present rat study, has shown that EE_2 may contribute to this induction. However, as the urinary excretion of the estradiol metabolites was not studied direct induction of the glucuronide pathway cannot be surmized. The impairment of estradiol oxidation which may have occurred in OCS users (see Chapter 5) was not discovered during chronic EE_2 pretreatment in female rats. This may have been due to the limitations of the study where changes in the phase I metabolism of estradiol would not be detected, or the oxidation of estradiol may not have been affected by EE_2 pretreatment. Further work needs to be performed in order to determine which component is responsible for decreasing oxidation of both exogenous and endogenous compounds.

Summary of the Effects of Oral Contraceptive Steroids

The present work has found that OCS use may result in either induction of the glucuronidation of paracetamol and endogenous estradiol or inhibition of the sulphation pathways of these 2 compounds. However, evidence from other workers indicates that induction of the glucuronidation pathway is more likely. Glycine conjugation was not found to be affected by OCS use, indicating that the contrasting results of other workers may be of little clinical significance. OCS use may also result in the inhibition of endogenous estradiol oxidation, which supports the results of other workers who found that OCS cause inhibition of the oxidation of several compounds, including exogenously administered estradiol.

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APPENDIX 1. Details of volunteers in aspirin study and the total amounts of aspirin metabolite excreted in a 0 - 8 hr urine following a therapeutic dose of aspirin (600 mg).

Volunteer	Sex	Age (yrs)	Body Weight (kg)	Smoker	Other drugs taken concomitantly with aspirin	Urine Volume (mls)	Urine pH	Amount recovered in 0-8 hr urine (mg)			SA(corrected SUA
								Observed SA	SA corrected to pH 6.2	SUA	
1	F	24	44	No	-	745	6.70	29.2	20.4	201.2	0.101
2	M	24	70	Yes	-	885	6.27	5.3	4.3	200.9	0.021
3	M	18	63	N/K	-	400	6.93	31.4	17.0	217.9	0.078
4	M	19	70	N/K	-	415	7.00	26.5	10.1	201.6	0.050
5	M	24	71	No	-	320	5.92	11.3	14.7	233.1	0.063
6	M	29	65	No	-	555	6.43	13.2	9.7	193.4	0.050
7	F	21	63	No	OCS	270	5.70	5.7	11.1	226.1	0.049
8	M	18	73	N/K	-	845	6.12	8.9	10.0	236.3	0.042
9	M	20	67	No	-	270	6.90	14.8	1.2	226.0	0.005
10	F	20	N/K	No	-	685	6.47	24.8	20.6	199.6	0.103
11	F	20	68	N/K	N/K	570	6.40	17.0	14.0	179.3	0.078
12	M	18	64	N/K	-	410	5.95	10.9	13.9	171.6	0.081
13	M	19	N/K	N/K	-	360	5.30	2.9	10.9	229.9	0.047
14	M	22	60	N/K	-	400	6.80	37.1	26.0	211.6	0.123
15	M	18	76	N/K	-	965	7.00	19.7	3.3	199.6	0.016
16	F	19	54	N/K	N/K	600	6.15	15.15	15.85	203.7	0.078
17	F	20	58	N/K	N/K	645	7.00	40.4	24.0	95.0	0.253
18	M	20	62	No	-	950	7.08	35.6	16.8	204.1	0.082

Appendix 1 continued

Volunteer	Sex	Age (yrs)	Body Weight (kg)	Smoker	Other drugs taken concomitantly with aspirin	Urine Volume (mls)	Urine pH	Amount recovered in 0-8 hr urine (mg)			SA(corrected)
								Observed SA	SA corrected to pH 6.2	SUA	SUA
19	M	21	73	No	-	1090	6.70	13.2	4.4	158.4	0.028
20	M	22	68	No	-	375	6.40	20.5	17.5	235.8	0.074
21	F	21	67	No	-	895	6.30	9.5	8.1	211.8	0.038
22	F	23	54	N/K	N/K	305	5.35	5.4	13.2	144.8	0.091
23	F	19	60	No	-	605	6.07	10.6	12.3	192.4	0.064
24	F	20	57	No	-	710	7.30	86.1	59.9	96.0	0.624
25	M	20	70	N/K	-	345	6.15	14.0	14.7	205.6	0.071
26	F	20	57	No	-	850	6.10	14.3	15.6	209.3	0.074
27	M	23	76	No	-	355	6.43	16.95	13.45	210.6	0.064
28	M	26	70	No	-	625	6.47	9.0	4.8	183.4	0.026
29	F	19	63	No	-	295	6.03	22.7	24.9	220.6	0.113
30	M	22	60	No	-	1055	6.70	19.25	10.45	224.8	0.046
31	F	23	57	No	OCS	595	5.95	13.6	16.6	164.0	0.101
32	F	23	60	N/K	N/K	155	5.97	3.8	6.6	206.3	0.032
33	F	36	90	N/K	N/K	280	6.00	12.9	15.4	237.4	0.065
34	F	29	64	N/K	N/K	230	7.00	19.9	18.9	211.1	0.089
35	M	53	95	N/K	-	390	6.00	15.15	17.6	255.4	0.069
36	M	38	68	N/K	-	895	6.50	20.3	15.5	153.8	0.101
37	M	26	76	No	-	1830	6.35	32.7	30.5	218.6	0.139
38	M	54	70	No	Oxytetracycline	310	5.23	1.1	9.5	197.0	0.048
39	M	21	67	No	-	400	5.27	3.0	11.2	269.0	0.041
40	F	21	48	No	OCS	250	6.40	24.15	21.15	168.0	0.126

Appendix 1 continued

Volunteer	Sex	Age (yrs)	Body Weight (kg)	Smoker	Other drugs taken concomitantly with aspirin	Urine Volume (mls)	Urine pH	Amount recovered in 0-8 hr urine (mg)			SA(corrected)
								Observed SA	SA corrected to pH 6.2	SUA	SUA
41	M	23	62	No	-	250	5.52	1.45	8.15	211.8	0.038
42	F	38	86	Yes	-	890	6.60	29.6	22.9	232.0	0.099
43	M	39	70	No	-	520	5.78	4.0	8.7	231.7	0.037
44	M	45	70	No	-	1260	6.25	7.9	7.2	227.2	0.032
45	M	48	86	No	-	1110	5.78	3.4	8.1	235.5	0.034
46	F	48	79	No	-	850	6.80	35.9	24.8	192.2	0.129
47	M	25	70	Yes	-	2970	6.20	12.5	12.5	250.9	0.050
48	M	57	68	Yes	-	890	6.75	11.7	1.8	179.7	0.010
49	M	34	N/K	Yes	-	2230	5.58	4.1	10.4	250.1	0.042
50	M	22	64	No	-	365	6.60	15.5	8.8	221.3	0.040
51	F	44	50	No	Tryptophan	1710	6.70	38.9	30.1	180.1	0.167
52	M	52	73	Yes	-	1190	5.38	0.6	8.2	173.1	0.047
53	M	61	73	No	-	610	5.77	7.5	12.3	202.9	0.061
54	M	44	71	No	-	900	5.72	12.5	17.7	223.7	0.079
55	M	39	65	No	Neonaclex	700	6.75	7.6	-2.3	166.1	-0.014
56	M	58	76	No	-	890	5.88	4.4	8.2	228.0	0.036
57	M	24	67	No	-	920	7.10	63.1	43.7	198.8	0.220
58	M	25	N/K	Yes	-	1650	5.30	0.0	8.0	239.1	0.033
59	M	29	73	No	-	720	6.30	17.8	16.4	235.1	0.070
60	M	24	67	No	-	490	6.10	6.5	7.8	269.2	0.029
61	M	40	67	No	-	540	5.95	8.7	11.7	191.1	0.061
62	F	45	62	No	-	450	5.07	0.0	14.7	51.2	0.287
63	M	29	75	Yes	-	3230	6.85	39.2	26.9	225.4	0.119

Appendix 1 continued

Volunteer	Sex	Age (yrs)	Body Weight (kg)	Smoker	Other drugs taken concomitantly with aspirin	Urine Volume (mls)	Urine pH	Amount recovered in 0-8 hr urine (mg)			SA(corrected)
								Observed SA	SA corrected to pH 6.2	SUA	SUA
64	F	59	57	No	Propranolol	480	5.20	2.5	11.0	41.5	0.265
65	M	22	63	Yes	Beconase	860	6.90	20.5	6.9	207.3	0.033
66	M	38	73	Yes	-	660	5.05	15.7	24.9	158.1	0.157
67	F	39	54	No	-	990	6.45	29.8	25.9	90.8	0.285
68	F	23	57	Yes	-	560	5.62	7.0	13.0	78.5	0.166
69	F	27	60	Yes	OCS	200	5.30	0.6	8.6	28.2	0.305
70	F	18	57	No	-	350	6.90	12.3	-1.3	50.1	-0.026
71	F	18	60	No	-	170	5.95	4.8	7.8	42.9	0.182
72	F	21	49	No	OCS	340	5.62	7.9	13.9	61.4	0.226
73	F	29	53	Yes	OCS	300	6.30	35.9	34.5	89.6	0.385
74	M	33	73	No	-	655	5.40	6.6	14.1	444.7	0.032
75	M	53	83	No	-	500	5.70	2.0	7.4	224.3	0.033
76	M	57	70	No	-	430	5.75	8.0	13.0	207.2	0.063
77	F	43	61	No	Vitamin B	950	6.75	73.4	63.5	200.1	0.317
78	F	51	70	No	-	650	6.20	15.2	15.2	197.0	0.077
79	F	22	61	No	-	235	5.45	2.6	9.8	169.9	0.058
80	M	54	87	No	Propanolol	950	6.40	2.4	-0.6	95.4	-0.006
81	M	54	N/K	No	Palvadrine	1380	6.35	16.0	13.8	215.9	0.064
82	M	25	N/K	No	-	720	5.40	2.3	9.8	318.1	0.031
83	M	35	95	No	-	800	5.72	8.4	13.6	255.7	0.053
84	M	44	78	No	-	960	6.70	19.1	10.3	244.1	0.095
85	F	47	57	Yes	-	660	5.95	36.1	39.2	195.5	0.200
86	M	35	75	Yes	Sodium chromoglycate	1040	6.30	3.2	-7.9	295.9	-0.027
87	F	18	N/K	No	-	220	6.75	33.0	23.1	210.2	0.110

Appendix 1 continued

Volunteer	Sex	Age (yrs)	Body Weight (kg)	Smoker	Other drugs taken concomitantly with aspirin	Urine Volume (mls)	Urine pH	Amount recovered in 0-8 hr urine (mg)			SA(corrected)
								Observed SA	SA corrected to pH 6.2	SUA	SUA
88	F	32	46	No	-	610	5.45	4.8	11.8	173.5	0.068
89	M	28	60	Yes	-	400	7.15	50.6	29.6	201.6	0.147
90	M	36	N/K	No	-	670	6.50	21.8	17.1	222.0	0.077
91	M	48	71	No	-	1290	6.45	15.5	11.6	181.3	0.064
92	M	28	64	Yes	-	800	6.50	23.2	18.5	200.8	0.092
93	M	45	60	Yes	-	480	5.70	10.6	16.0	246.2	0.065
94	M	48	70	Yes	-	790	5.40	3.7	11.2	240.6	0.046
95	M	50	N/K	No	-	1440	5.40	6.2	13.7	240.9	0.057
96	F	54	54	No	-	1240	6.10	38.6	39.9	209.6	0.190
97	M	38	70	No	-	1040	6.02	13.3	15.6	219.2	0.071
98	F	23	55	No	-	790	6.45	36.4	32.5	191.7	0.169
99	M	37	77	No	-	540	6.65	39.2	31.5	191.5	0.165
100	M	52	76	No	Ventolin, Becotide, Intal	815	5.80	11.4	15.9	199.1	0.080
101	M	56	70	No	-	370	6.60	46.6	39.9	122.7	0.325
102	M	51	79	No	-	440	5.79	8.8	13.4	280.1	0.048
103	M	33	75	Yes	-	440	6.75	59.6	49.7	225.7	0.220
104	M	55	78	No	-	890	5.55	10.1	16.6	199.5	0.083
105	M	43	84	No	-	490	5.82	14.6	18.9	242.1	0.078
106	M	44	89	Yes	-	330	5.30	4.1	12.1	226.7	0.053
107	M	34	89	Yes	-	1790	6.05	11.3	13.2	265.6	0.050
108	M	59	78	Yes	-	730	5.30	6.3	14.3	209.6	0.068
109	M	42	102	No	-	2270	5.15	2.6	11.3	273.9	0.041
110	M	22	97	Yes	-	1120	6.75	23.2	13.3	167.2	0.079
111	M	21	N/K	Yes	-	590	7.20	22.9	0.2	175.9	0.001

Appendix 1 continued

Volunteer	Sex	Age (yrs)	Body Weight (kg)	Smoker	Other drugs taken concomitantly with aspirin	Urine Volume (mls)	Urine pH	Amount recovered in 0-8 hr urine (mg)			SA(corrected)
								Observed SA	SA corrected to pH 6.2	SUA	SUA
112	F	19	57	No	-	390	6.01	9.0	11.4	165.75	0.068
113	F	21	77	No	-	590	6.74	8.2	-1.5	72.9	-0.021
114	F	22	64	No	OCS	360	6.35	10.8	8.6	316.5	0.027
115	F	19	60	No	-	290	6.78	8.5	-2.1	67.9	-0.031
116	F	21	60	No	-	310	6.50	19.1	14.4	345.6	0.042
117	F	N/K	49	No	-	270	7.19	47.3	26.3	159.1	0.165
118	F	28	57	No	-	280	5.38	8.2	15.8	179.9	0.088
119	M	19	75	No	-	470	5.26	4.75	12.95	160.1	0.081
120	F	19	57	No	-	350	6.28	12.95	11.85	311.2	0.038
121	F	46	74	No	-	250	5.86	7.15	11.15	113.0	0.099
122	M	19	63	Yes	Minocin	200	7.28	31.0	5.5	92.5	0.059
123	F	19	N/K	No	-	540	5.87	7.5	11.4	90.9	0.125
124	M	24	N/K	Yes	-	330	6.70	7.2	-1.6	129.3	-o.012
125	F	18	70	No	-	850	7.52	32.3	-3.0	144.1	-0.208
126	F	28	56	No	Vitamin B_{12}	550	7.33	39.3	11.9	134.5	0.088
127	F	21	70	No	-	370	5.65	0.0	5.8	120.8	0.048
128	F	19	53	No	-	260	7.42	30.5	-0.5	111.0	-0.004
129	F	22	61	No	-	410	6.94	10.8	-3.9	133.2	-0.029
130	F	20	57	Yes	-	250	8.02	34.2	-29.9	101.6	-0.294
131	M	22	68	No	-	890	6.46	34.7	30.7	227.4	0.135
132	M	22	76	No	-	430	6.27	15.6	14.6	210.7	0.069
133	F	19	54	No	OCS	650	6.49	29.8	25.2	206.7	0.122
134	M	19	70	No	-	260	7.96	44.1	-14.8	198.9	-0.074

Appendix 1 continued

Volunteer	Sex	Age (yrs)	Body Weight (kg)	Smoker	Other drugs taken concomitantly with aspirin	Urine Volume (mls)	Urine pH	Amount recovered in 0-8 hr urine (mg)			SA(corrected)
								Observed SA	SA corrected to pH 6.2	SUA	SUA
135	M	18	70	No	-	140	7.34	8.9	-18.9	88.8	-0.213
136	M	20	67	No	-	400	6.31	16.6	15.0	229.2	0.065
137	M	20	92	No	-	540	6.67	18.3	10.2	240.3	0.042
138	F	18	63	No	-	380	6.64	37.1	29.6	211.5	0.140
139	M	19	79	Yes	-	300	6.62	10.0	2.9	220.6	0.013
140	M	18	63	No	Oxytetracyclin	420	5.85	12.2	16.3	184.2	0.088
141	F	22	62	No	-	1580	7.12	60.2	39.2	217.2	0.180
142	M	23	72	No	-	860	7.00	9.2	-7.2	212.4	-0.034
143	F	27	53	No	-	1120	6.29	19.8	18.5	198.2	0.093
144	F	41	70	No	-	250	5.09	2.4	11.4	200.6	0.057
145	F	25	63	No	OCS	200	5.63	4.4	10.3	199.7	0.052
146	F	27	66	No	-	570	6.16	19.5	20.1	197.8	0.102
147	F	20	57	No	OCS	490	5.71	16.4	21.7	132.4	0.164
148	F	39	47	Yes	-	1350	6.34	38.8	36.8	222.75	0.165
149	M	53	N/K	No	-	610	5.5	7.4	14.3	226.6	0.063
150	M	N/K	N/K	N/K	N/K	560	5.9	12.5	16.1	190.1	0.085

APPENDIX 1a. Repeat aspirin study: Total amounts of aspirin metabolites excreted in 0 - 8 hr urine following 2 separate administrations of aspirin (the therapeutic dose of 600 mg ASA given on each occasion).

Volunteer	Time between repeat tests (months)	FIRST TEST					SECOND TEST				
		Urine Vol	Urine pH	Observed SA(mg)	Corrected SA (mg)	SUA (mg)	Urine Vol	Urine pH	Observed SA(mg)	Corrected SA (mg)	SUA (mg)
7	17	270	5.70	5.7	11.1	226.1	240	6.59	32.6	26.1	185.3
10	27	685	6.47	24.8	20.6	199.6	770	6.76	68.3	58.2	191.0
22	17	305	5.35	5.4	13.2	144.8	320	6.81	73.7	62.4	168.7
24	26	710	7.30	86.1	59.9	96.0	330	5.59	13.0	19.2	140.0
27	16	355	6.43	16.95	13.45	210.6	390	6.27	17.9	16.9	221.5
32	15	155	5.97	3.8	6.6	206.3	880	6.43	3.6	0.1	81.3
41	7	250	5.52	1.45	8.15	211.8	2450	6.74	17.6	7.9	224.5
43	7	520	5.78	4.00	8.7	231.7	820	5.85	5.2	9.3	240.0
55	7	700	6.75	7.6	-2.3	166.1	700	6.77	9.7	-0.7	205.8
59	7	490	6.10	6.5	7.8	235.1	390	5.68	8.7	14.2	109.8
66	19	660	5.05	15.7	24.9	158.1	600	4.97	3.5	12.95	245.7
84	18	1040	6.80	3.2	-7.9	244.1	1000	6.97	43.7	28.2	252.0
100	18	540	6.65	39.2	31.5	191.5	270	6.15	14.7	15.4	188.7

APPENDIX 2. Details of volunteers in paracetamol study and the total amounts of paracetamol metabolites excreted in a 0 - 8 hr urine following a therapeutic dose of paracetamol (1 g)

Volunteer	Sex	Age (yrs)	Body Weight (kg)	Smoker	Other drugs taken concomitantly with paracetamol	Urine Volume (mls)	Amount excreted in 0-8 hr urine (mg)			
							PG	PS	P	P + PG + PS
1	F	27	48	No	-	140	414.5	515.8	8.5	938.8
2	F	43	52	Yes	-	290	715.4	236.8	14.6	966.8
3	M	37	68	No	-	1240	599.5	236.2	29.7	865.4
4	M	29	65	Yes	-	2330	433.4	224.8	30.5	688.7
5	M	44	87	No	-	1000	323.0	257.0	35.9	615.9
6	F	26	46	No	-	200	433.3	240.9	6.8	681.0
7	M	37	76	Yes	-	510	504.1	348.3	20.1	872.4
8	F	47	78	Yes	-	1170	420.0	267.3	30.5	717.8
9	M	41	84	No	-	1150	286.3	159.3	22.5	468.1
10	F	40	51	No	Fe_2SO_4	490	238.9	118.3	11.6	368.8
11	M	36	86	Yes	-	1360	261.1	182.9	13.4	457.4
12	F	31	61	No	Anafranil	1020	458.5	93.3	14.9	566.7
13	M	43	67	Yes	-	210	271.4	131.5	11.1	414.0
14	M	19	79	No	-	1130	533.4	55.9	16.5	605.7
15	M	22	N/K	No	-	2050	317.7	71.7	11.1	400.5
16	M	34	79	No	-	1850	371.9	86.0	8.4	466.3
17	M	59	76	Yes	-	890	396.9	122.4	17.5	538.8
18	M	25	48	No	-	1320	362.3	142.6	17.3	522.7
19	M	34	59	No	-	600	324.0	150.9	14.6	489.5

Appendix 2 continued

Volunteer	Sex	Age (yrs)	Body Weight (kg)	Smoker	Other drugs taken concomitantly with paracetamol	Urine Volume (mls)	Amount excreted in 0-8 hr urine (mg)			
							PG	PS	P	P + PG + PS
20	F	57	59	No	-	410	302.0	168.9	17.8	488.7
21	M	25	N/K	No	-	1550	317.8	71.3	17.4	406.5
22	M	54	79	No	Atenolol	510	194.8	90.0	13.1	297.9
23	F	24	N/K	No	OCS	580	346.0	152.0	19.5	517.5
24	M	20	73	No	-	2100	355.9	200.5	36.8	593.2
25	M	46	70	No	-	2060	443.9	167.9	23.7	635.5
26	F	27	54	No	-	1030	526.3	250.3	20.4	797.0
27	F	25	67	No	-	700	410.2	177.8	14.5	602.5
28	M	36	78	No	-	570	471.7	157.9	14.0	643.6
29	M	23	73	No	-	670	301.2	135.7	16.8	453.7
30	F	31	N/K	No	-	810	518.8	203.7	33.6	756.1
31	M	36	71	No	-	200	461.8	238.8	13.4	714.0
32	M	39	64	Yes	-	380	301.0	245.5	17.2	563.7
33	M	38	82	No	-	300	404.25	262.0	15.0	681.25
34	M	39	N/K	Yes	-	980	444.4	392.0	25.7	862.1
35	M	38	73	No	-	700	587.7	162.4	21.0	771.1
36	M	42	74	No	Diuretic or blocker or placebo	1130	431.1	284.2	17.6	732.9
37	F	47	64	No	Diuretic	310	388.0	276.2	24.9	689.1
38	M	47	87	No	-	340	268.4	292.6	21.8	582.8
39	F	27	N/K	No	OCS	240	516.5	93.0	11.8	621.3
40	M	45	N/K	No	Amitryphiline; Nitrazolam	810	491.3	181.0	20.0	620.3

Appendix 2 continued

Volunteer	Sex	Age (yrs)	Body Weight (kg)	Smoker	Other drugs taken concomitantly with paracetamol	Urine Volume (mls)	Amount excreted in 0-8 hr urine (mg)			
							PG	PS	P	P + PG + PS
41	M	21	76	No	-	540	509.2	161.5	14.2	684.9
42	F	51	58	No	-	450	294.1	233.3	30.2	557.6
43	M	46	N/K	No	-	750	320.6	180.4	12.3	513.3
44	M	39	72	No	Clonidene	1210	346.1	197.2	20.1	563.4
45	M	44	N/K	No	-	660	345.5	173.9	18.8	538.2
46	M	47	71	No	-	1420	474.3	182.5	24.5	681.3
47	M	37	83	No	-	1110	505.0	245.9	26.4	776.3
48	M	54	74	No	-	670	376.9	380.2	26.5	783.6
49	F	53	70	No	-	580	370.3	289.4	20.5	680.2
50	M	39	75	No	-	370	587.0	205.2	31.8	824.0
51	M	20	102	No	-	610	526.7	221.7	24.8	773.2
52	M	24	65	No	-	335	419.1	288.1	26.8	734.0
53	M	59	78	No	-	440	362.8	290.2	7.3	660.3
54	M	46	84	Yes	-	1395	263.6	283.2	44.8	591.6
55	F	23	76	No	OCS	620	407.3	114.1	9.9	531.3
56	M	46	73	No	Bendrofluazide, Sanomigran	1335	571.4	200.2	18.9	790.5
57	F	29	54	No	-	1000	639.0	244.0	26.3	909.3
58	M	37	85	No	-	1320	462.7	215.8	24.4	702.9
59	M	26	87	No	-	505	406.0	177.0	12.9	595.9
60	M	35	72	No	Prednisolone	1450	395.1	289.3	24.0	708.4
61	F	19	61	No	OCS	385	418.9	293.0	3.7	715.6
62	M	56	N/K	No	-	660	295.0	208.9	5.6	509.5

Appendix 2 continued

Volunteer	Sex	Age (yrs)	Body Weight (kg)	Smoker	Other drugs taken concomitantly with paracetamol	Urine Volume (mls)	Amount excreted in 0-8 hr urine (mg) PG	PS	P	P + PG + PS
63	M	41	72	No	-	710	406.5	264.1	23.2	693.8
64	M	43	76	Yes	-	180	457.0	330.75	12.3	800.05
65	M	19	95	Yes	-	390	453.8	175.9	18.8	648.5
66	F	36	N/K	No	Ibuoprofen	570	523.8	247.4	12.6	783.8
67	F	40	95	Yes	-	950	570.5	560.5	21.0	1152.0
68	F	21	51	No	OCS	235	290.5	96.7	14.6	401.8
69	F	56	49	Yes	-	560	418.9	312.5	22.6	754.0
70	M	25	59	Yes	-	425	438.4	125.8	27.0	591.2
71	M	47	95	No	-	680	278.8	318.6	22.5	619.9
72	M	23	73	No	-	840	470.4	185.6	29.5	685.5
73	M	27	70	Yes	-	2330	475.3	143.3	25.9	644.5
74	M	20	74	No	-	780	481.3	256.6	40.0	778.0
75	M	54	83	Yes	-	740	558.7	199.8	20.9	779.4
76	F	46	51	No	-	630	323.1	272.3	22.1	617.5
77	M	62	64	No	-	935	207.1	233.75	46.2	487.05
78	M	18	N/K	No	-	360	434.5	144.0	26.7	605.2
79	M	28	N/K	Yes	-	1310	446.7	243.7	24.4	268.1
80	M	22	N/K	Yes	-	630	362.2	277.2	26.0	665.4
81	M	44	87	No	Tenormin,Apresoline	690	372.6	287.4	29.0	689.0
82	M	53	94	No	Tenormin, Histamet, Buprofen	840	494.3	160.4	17.4	672.1

Appendix 2 continued

Volunteer	Sex	Age (yrs)	Body Weight (kg)	Smoker	Other drugs taken concomitantly with paracetamol	Urine Volume (mls)	Amount excreted in 0-8 hr urine (mg)			
							PG	PS	P	P + PG + PS
83	M	55	67	No	Metoprolol	470	427.5	280.1	27.3	734.9
84	M	23	73	No	-	460	285.0	309.8	20.7	615.5
85	F	26	58	No	-	620	479.9	257.3	22.0	759.2
86	M	24	80	Yes	-	560	314.7	178.6	11.7	505.0
87	F	28	54	No	OCS	350	386.7	190.2	27.9	604.8
88	F	32	70	No	OCS	450	607.7	172.6	18.9	799.2
89	M	20	54	No	-	500	532.5	330.5	17.8	880.8
90	M	44	67	Yes	-	245	517.9	115.5	13.2	646.6
91	F	45	64	No	-	190	417.3	262.4	8.9	688.6
92	F	26	60	No	OCS	660	610.2	188.8	24.5	823.5
93	F	24	67	No	OCS	900	485.1	211.5	27.2	723.8
94	M	27	60	No	-	1000	572.5	248.5	32.4	853.4
95	F	39	47	Yes	-	1050	573.8	351.7	60.5	986.0
96	M	36	60.5	Yes	-	820	319.8	170.1	26.4	516.3
97	M	25	69	No	-	500	664.0	238.5	14.9	917.4
98	F	24	70	No	OCS	250	551.2	130.1	21.3	702.6
99	F	33	62	No	OCS	350	702.8	135.8	12.4	851.0

APPENDIX 3. Volunteer details for the oral contraceptive study and the 0 - 24 hour urinary excretion of endogenous estradiol and its phase II metabolites.

Volunteer	Age (yrs)	Body weight (kg)	Oral Contraceptive Steroid	Length of OCS use	Day of cycle	Smoker	Other drugs taken during study period	Plasma E_2 concn. (pg/ml)	Urine Volume (mls)	0-24 hr urinary excretion (ng) E_2	E_2G	E_2S
1	20	75	-	-	21	No	-	-	240	85.8	1245.0	445.3
2	20	61	Ovranette	10 months	16	Yes	-	17.8	800	167.8	364.9	33.9
3	21	44	Ovranette	3½ years	19	Yes	-	21.7	1590	120.8	91.5	9.2
4	21	64	-	-	16	No	-	108.7	1250	188.0	2584.4	1194.3
5	21	65	Norimin	4 years	19	No	-	17.4	995	117.4	363.8	36.9
6	21	60	Logynon	3 years	19	No	-	14.2	570	97.2	201.2	N/D
7	19	60	Brevinor	14 months	16	Yes	-	21.1	1180	76.5	235.3	42.5
8	21	56	-	-	38	No	-	256.2	2240	342.7	3577.3	2203.6
9	20	57	Binovum	9 months	18	No	-	26.3	725	164.9	318.7	N/D
10	22	63	Ovranette	1 year	18	No	-	18.1	1030	56.7	307.0	42.7
11	22	61	Trinordial	6 months	18	No	-	20.2	600	78.45	181.5	13.4
12	22	62	Logynon	1 year	20	No	-	17.3	690	149.9	449.2	5.2
13	20	53	Marvelon	10 months	18	No	-	15.0	720	149.7	172.5	N/D
14	20	69	Binovum	1 year	18	No	-	17.1	820	105.5	448.2	178.3
15	19	63	Ovranette	21 months	19	No	-	18.3	740	112.1	478.1	N/D
16	19	50	Trinordial	8 months	19	No	-	26.5	620	126.7	357.2	N/D
17	20	61	Microgynon	1½ years	18	Yes	-	23.5	1400	137.6	262.2	96.7
18	20	66	Logynon	3 years	17	Yes	-	17.1	690	75.2	147.4	N/D
19	19	62	Ovranette	2 years	20	No	-	17.8	1320	121.2	466.5	105.9
20	26	53	Microgynon	2½ years	19	No	-	22.2	1610	126.5	2437.2	4431.5

Appendix 3 continued

Volunteer	Age (yrs)	Body weight (kg)	Oral Contraceptive Steroid	Length of OCS use	Day of cycle	Smoker	Other drugs taken during study period	Plasma E_2 concn. (pg/ml)	Urine Volume (mls)	0-24 hr urinary excretion (ng) E_2	E_2G	E_2S
21	24	53	Trinordial	1 year	19	No	-	15.7	2220	162.5	2752.6	3839.2
22	22	72	-	-	18	No	-	51.1	2230	132.7	2136.8	584.7
23	22	63	-	-	21	No	Atenolol	208.5	1120	317.1	3932.6	1082.8
24	32	57	-	-	21	No	-	148.9	930	377.1	3442.8	1244.1
25	28	53	Microgynon	5 years	22	No	-	20.0	1450	66.5	1555.1	455.9
26	21	61	Microgynon	3 years	20	No	-	22.2	1400	283.2	434.0	266.7
27	19	54	-	-	20	No	-	136.0	960	251.4	1673.6	549.5
28	21	57	Logynon	3 years	19	No	-	15.1	570	122.7	233.1	N/D
29	21	75	Ovranette	5 years	20	No	-	26.0	1330	257.5	795.1	285.6
30	22	57	Microgynon	4 years	19	No	-	17.3	1150	183.5	442.6	26.4
31	21	66	Ovysman	2 years	19	No	-	14.3	1050	65.5	476.4	62.4
32	19	55	Logynon	2 years	20	No	-	18.6	1380	165.6	289.4	37.5
33	22	48	Brevinor	2 years	20	No	-	16.7	1690	109.2	298.4	139.4
34	18	55	Trinovium	7 months	19	No	-	15.6	720	91.8	101.8	2.7
35	19	55	Logynon	3 years	18	No	-	-	1580	130.0	226.9	N/D
36	19	57	Logynon	2 years	20	No	-	19.3	850	135.8	309.7	N/D
37	19	62	Trinordial	1 year	19	Yes	-	19.8	1480	217.1	304.5	94.0
38	19	66	Logynon	1 year	19	No	-	17.5	700	116.0	127.0	N/D
39	24	69	Logynon	8 years	17	No	-	-	1470	177.7	861.9	85.0
40	25	68	Microgynon	8 years	21	No	-	21.4	1460	298.9	243.2	N/D
41	20	63	Ovysmen	15 months	19	No	-	14.4	1120	80.8	187.7	164.2
43	21	57	Ovranette	3 years	20	No	-	18.8	1790	98.8	689.9	191.2
44	25	53	-	-	20	Yes	-	69.7	3360	2994.4	5484.9	1987.1
45	28	67	Brevinor	6 months	21	No	-	17.4	2560	243.3	567.3	52.2
46	32	68	Logynon	14 years	18	No	-	15.1	1700	1797	1359.7	238.6

APPENDIX 4. The 0 - 8 hour and the 8 - 24 hour urinary excretion of paracetamol and its major metabolites following an oral dose of paracetamol (50 mg/kg) in the female rat. (Urinary excretion expressed as % of administered dose).

	0 - 8 hr urine			8 - 24 hr urine		
	PG	PS	P			
EE_2-Treated Rats						
1T	23.5	65.3	2.6	-	-	-
2T	13.1	45.7	1.2	5.6	21.2	0.8
3T	13.4	33.6	1.8	1.4	12.9	3.2
4T	16.0	44.4	1.7	5.9	20.6	0.6
5T	15.9	50.6	1.7	0.9	12.3	2.8
6T	10.2	37.9	1.5	1.5	6.9	0.5
7T	25.5	54.7	2.6	2.5	8.1	0.6
8T	24.2	62.2	3.8	0.7	6.4	1.4
9T	11.1	35.5	1.5	1.5	5.2	0.2
10T	6.3	22.9	1.0	2.9	12.0	0.9
11T	19.2	40.9	2.0	6.0	20.7	0.6
12T	13.6	48.1	2.2	2.5	9.2	1.0
Control Rats						
1C	18.4	51.3	2.4	2.3	15.6	2.1
2C	13.7	65.6	2.0	2.2	14.2	0.4
3C	13.3	48.9	2.1	3.2	19.5	0.6
4C	9.6	37.2	1.5	4.8	29.9	0.8
5C	5.8	28.9	1.7	4.2	37.7	0.7
6C	9.9	32.0	1.9	0.9	16.4	3.9
7C	12.5	42.9	1.4	0.8	12.9	2.5
8C	8.4	41.2	1.5	2.3	14.1	1.0
9C	9.8	41.6	1.6	3.0	16.0	1.1
10C	12.3	38.2	2.3	3.2	11.9	1.1
11C	15.1	46.1	1.9	2.3	17.0	0.7
12C	4.7	17.9	1.6	5.0	23.7	0.5

APPENDIX 5. 24 hour urinary excretion of total estradiol and 24 hour estradiol content of several tissues following IV administration of ^{14}C-E_2 (26.6 g; 5.07 Ci/kg) in the female rat.

RAT	Total Radioactivity in 0-24 hr urine ($CPM \times 10^3$)	Tissue radioactive content 24 hours after ^{14}C-E_2 administration ($CPM \times 10^3$)/g tissue)			
		Liver	Small Intestine	Large Intestine	Faeces
EE_2-Treated Rats					
1T	27.80	1.96	29.06	61.23	26.96
2T	39.62	1.79	29.00	134.49	23.79
3T	28.67	3.36	38.07	65.50	251.25
4T	37.82	2.05	13.30	69.06	484.36
5T	34.93	2.37	33.89	103.04	7.94
Control Rats					
1C	20.92	3.01	118.72	84.44	21.24
2C	18.73	2.60	45.59	120.91	21.96
3C	16.47	2.45	51.75	100.07	19.83
4C	19.87	3.21	41.46	169.77	94.29
5C	22.48	2.93	53.88	105.32	-